Einspruch gegen ein europäisches Patent

An das Europäische Patentamt

Tabulatoren-Positionen

nur für EPA

I. Angegriffenes Patent

Einspr.-Nr. OPPO (1)

Patentnummer	EP 0 656 786
Anmeldenummer	93909679.8
Tag des Hinweises auf Erteilung (Art.97(4), 99(1) EPÜ)	15.09.2004

Bezeichnung der Erfindung (Titel): Verwendung von Isoflavon Phyto-Östrogen Extrakten von Soja oder Klee

II. Patentinhaber

In der Patentschrift als erster/einziger genannter
KELLY, Graham Edmund, Northbridge, NSW 2063 (AU)

Zeichen des Einsprechenden oder Vertreters (maximal 15 Positionen): XI 766/05 OREF

III. Einsprechender

OPPO (2)

Name: Jukunda Naturarzneimittel
Dr. Ludwig Schmitt GmbH & Co. KG

Anschrift: Hofmarkstr. 35
82152 Planegg

EPO -DG 1
15. 06. 2005
114

Staat des Wohnsitzes oder Sitzes: DEUTSCHLAND

Telefon/Telex/Telefax:

Gemeinsamer Einspruch: ☐ Miteinsprechende siehe Zusatzblatt

IV. Bevollmächtigung

1. **Vertreter** (Nur einen Vertreter angeben, dem zugestellt werden soll)

OPPO (9)

Rechtsanwalt Christlieb Klages

Name / Geschäftsanschrift:
HERTIN
Anwaltssozietät
Kurfürstendamm 54/55
10707 Berlin
Deutschland

Telefon/Telex/Telefax: +49-30-885 929-0 +49-30-885 929-29

Weitere zugelassene Vertreter: ☐ (siehe Zusatzblatt/Vollmacht) OPPO (5)

2. **Angestellte(r)** des Einsprechenden, die/der für dieses Einspruchsverfahren gemäß Art. 133(3) EPÜ bevollmächtigt werden/wird: Name(n):

Vollmacht(en): ☐ nicht erforderlich

Zu 1./2.: ☐ registriert unter Nr. ☐ beigefügt

EPA Form 2300.1 04.93

chèque : EUR 610
ZUR KASSE

nur für EPA

V. Der Einspruch richtet sich gegen das erteilte Patent

— im gesamten Umfang [X]

— im Umfang der Ansprüche Nr. [ ]

VI. Einspruchsgründe:

Der Einspruch wird darauf gestützt, daß

(a) der Gegenstand des europäischen Patents nicht Patentfähig ist (Art. 100(a) EPÜ), weil er

— nicht neu ist (Art. 52(1); 54 EPÜ) [X]

— nicht auf einer erfinderischen Tätigkeit beruht (Art. 52(1): 56 EPÜ) [X]

— aus sonstigen Gründen nämlich [Art. 52 (4)] [X]

von der Patentierbarkeit ausgeschlossen ist.

(b) das europäische Patent die Erfindung nicht so deutlich offenbart, daß ein Fachmann sie ausführen kann (Art. 100(b) EPÜ, vgl. Art. 83 EPÜ). [X]

(c) der Gegenstand des europäischen Patents über den Inhalt der Anmeldung/der früheren Anmeldung in der ursprünglich eingereichten Fassung hinausgeht (Art. 100(c) EPÜ, vgl. Art. 123(2) EPÜ). [ ]

VII. Tatsachenvorbringen und Begründung

(Regel 55(c) EPÜ)
erfolgt auf gesondertem Schriftstück (Anlage 1) [X]

VIII. Sonstige Anträge:

Hilfsweise wird mündliche Verhandlung beantragt

IX. Beweismittel

nur für EPA

Beigeschlossen = X

wird / werden nachgereicht =

A Veröffentlichungen:

Datum der Veröffentlichung

1

Conference Report: Consensus Development Converence: Prophylaxis and Treatment of Osteoporosis, January 1991 The American Journal of Medicine Volume 90: 107-110

Besonders relevant (Seite/Spalte/Zeile/Fig.):

2

Commentary: The Role of Soy Products in Reducing Risk of Cancer, Mark Messina, Stephen Barnes, Journal of the National Cancer Institute, Vol. 83, No. 8, April 17, 1991: 541 - 546

Besonders relevant (Seite/Spalte/Zeile/Fig.):

3

Naturally occurring oestrogens in foods – A review, K. R. Price and G. R. Fenwick, Food Additives and Contaminants, 1985, Vol. 2, No. 2, 73-106

Besonders relevant (Seite/Spalte/Zeile/Fig.):

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Use of a Mammalian Cell Culture Benzu(a)pyrene Metabolism Assay for the Detection of Potential Anticarcinogens from Natural Products: Inhibition of Metabolism by Biochanin A, an Isoflavone from Trifolium pretense L[1], John M. Cassady, Thomas M. Zennie, Young-Heum Chae, Mark A. Ferin, Nuris E. Portuondo, and William M. Baird, Cancer Research 48, 6257-6261, November 15, 1988

Besonders relevant (Seite/Spalte/Zeile/Fig.):

5

Short Paper, Oestrogenic Activity of Soya-Bean Products, H. M. Drane, D. S. P. Patterson, B. A. Roberts and N. Saba, Central Veterinary Laboratory, Weybridge, Surry, KT15 3NB, England, Received 22 October 1979, Fd Cosmet, Toxicol. Vo. 18, pp 425-427

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6

Deutsche Tierärztliche Wochenschrift Nr. 11, Oestrogenwirksame Isoflavone in Trifolium pretense (Rotklee), Verteilung in den oberirdischen Pflanzenteilen und Vorkommen als "gebundene" Isoflavone, von G. Schultz – Mit 3 Abbildungen, aus dem Botanischen Institut der Tierärztlichen Hochschule Hannover, Direktor: Professor Dr. E. Perner: 246-251

Besonders relevant (Seite/Spalte/Zeile/Fig.):

7

Nonsteroidal estrogenes of dietary origin: possible roles in hormone-dependent disease, KDR Setchell, SP Borriello, P Hulme, DN Kirk, and M Axelson, The American Journal of Clinical Nutrition 40: September 1984, pp 569-578. Printed in USA 1984 American Society for Clinical Nutrition

Besonders relevant (Seite/Spalte/Zeile/Fig.):

Fortsetzung auf Zusatzblatt X

B. Sonstige Beweismittel

Weitere Angaben auf Zusatzblatt

IX. Beweismittel

nur für EPA

Beigeschlossen = X

wird / werden nachgereicht =

A **Veröffentlichungen:** — Datum der Veröffentlichung

8
Deutsche Tierärztliche Wochenschrift Nr. 17, Isoflavone in einigen Weiß- und Rotkleesorten und ihre oestrogene Wirksamkeit bei juvenilen Mäusen, von E. Grunert und Gudrun Woelke, aus der Klinik für Geburtshilfe und Gynäkologie des Rindes der Tierärztlichen Hochschule Hannover, Direktor: Prof. Dr. E. Aehnelt und G. Schultzs, aus dem Botanischen Institut der Tierärztlichen Hochschule Hannover – Direktor: Prof. Dr. E. Perner. 1. September, 431-433

Besonders relevant (Seite/Spalte/Zeile/Fig.):

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Soya – a dietary source of the non-steroidal oestrogen equol in man and animals. M. Axelson, J. Sjövall, B. E. Gustafsson and K. D. R. Setchell, Received 21 October 1983, J. Endocr. (1984) 102, 49-56, 1984 Journal of Endocrinology Ltd, Printed in Great Brit- ain, 0022-0795/84/0102-0049 $02.00/0

Besonders relevant (Seite/Spalte/Zeile/Fig.):

10
Western diet and Western diseases: some hormonal and biochemical mechanismus and associations, Herman Adlercreutz, Department of Clinical Chemistry, University of Helsinki, Meilahti Hospital, Helsinki, Finnland, Scand J Clin Lab Invest 1190; 50, Suppl 201: 3-23

Besonders relevant (Seite/Spalte/Zeile/Fig.):

11
Acta vet. scand. 1987, 28, 255-262. From the Department of Animal Hygiene, College of Veterinary Medicine, Helsinki and The Agricultural Research Centre, South Savo Research Station, Mikkeli, Finnland.: Variations in the Content of Plant Oestrogens in the Red Clover-Timothy-Grass during the Grwing Season. By K. Kallela, I. Saastamoinen and E. Huokuna. Acta vet. scand. vol. 28 no. 3-4 - 1987

Besonders relevant (Seite/Spalte/Zeile/Fig.):

12
Sexual behaviour of ewes with clover disease treated repeatedly with oestradiol benzoate or testosterone propionate after ovariectomy, N. R. Adams, CSIRP Division of Animal Production, Private Bag, Wembley, Western Australia 6014, J. Reprod. Fert. (1983) 68. 113-117, Printed in Great Britain, 0022-4251/83/030113-05$02-00/0, 1983 Journals of Reproduction & Fertility Ltd.

Besonders relevant (Seite/Spalte/Zeile/Fig.):

13
The Oestrogenic Activity of red clover Isoflavones and some of their Degradation Products, E. Wong and D. S. Flux, Received 28 December 1961, J. Endocrin. (1962), 24, 341-348, Printed in Great Britain
Besonders relevant (Seite/Spalte/Zeile/Fig.):

14
Determination of Urinary Lignans and Phytoestrogen Metabolites, Potential Antiestrogens and Anticarcinogens, in Urine of women on various habitual diets, H. Adelcreutz, T. Fotsis, C. Bannwart, K. Wähälä, T. Mäkelä, G. Brunow and T. Hase, Proceedings of the XII International Study Group for Steroid Hormones (Rome, 2-4 December 1985), J. steroid Biochem. Vo. 25, No. 5B, pp. 791-797, 1986, Printed in Great Britain. 0022-4731/86 $3.00 + 0.00, 1986 Pergamon Journals Ltd.
Besonders relevant (Seite/Spalte/Zeile/Fig.):

Fortsetzung auf Zusatzblatt X

B. **Sonstige Beweismittel**

Weitere Angaben auf Zusatzblatt ☐

IX. Beweismittel

nur für EPA

Beigeschlossen = [X]

wird / werden nachgereicht = [ ]

A **Veröffentlichungen:** | Datum der Veröffentlichung

15
V/ Occurrence of Anabolic Agents in Plants and their Importance, H. R. Lindner, Department of Hormone Research, The Weizmann Institute of Science, Rehovot, Israil, 151-159
Besonders relevant (Seite/Spalte/Zeile/Fig.):

16
Urinary excretion of lignangs and isoflavonoid phytoestrogens in Japanese men and women consuming a traditional Japanese diet. Herman Adelcreutz, Hideo Honjo, Akane Higashi, Theodore Fotsis, Esa Hämäläinen, Takeshi Hasegawa, and Hiroji Okada, Am J Clin Nutr 1991; 54:1093-1100. Printed in USA. 1991 American Society for Clinical Nutrition

Besonders relevant (Seite/Spalte/Zeile/Fig.):

17
The Use of Thermospray Liquid Chromatography/ Tandem Mass Spectrometry for the Class Identification and Structural Verification of Phytoestrogens in Soy Protein Preparations, Robert J. Barbuch und John E. Coutant, Mary Beth Welsh and K. D. R. Setchell, Biomedical and Environmental mass spectrometry, Vol. 18. 973-977 (1989), Received 13 April 1989, Accepted (revised) 3 July 1989

Besonders relevant (Seite/Spalte/Zeile/Fig.):

18
Short Papers: Oestrogenic Response of the CD-1 Mouse to the Soya-Bean Isoflavones Genistein, Genistin and Daidzin, E. Farmakalidis and P. A. Murphy, Department of Food Technology, Iowa State University, Ames, IA 50011, USA, Received 1 October 1983, Fa Chem. Toxic. Vol. 22, no. 3, pp. 237-239, 1984, Printed in Great Britain
Besonders relevant (Seite/Spalte/Zeile/Fig.):

19
Isoflavone Content of Soya-Based Laboratory Animal diets, P. A. Murphy, E. Farmakalidis and L. C. Johnson, Department of Food Technology, Iowa State University, Ames, I A 50011, USA, Received 4 August 1981, Fd Chem Toxic, Vol. 20. pp. 315 to 318, 1982, Printed in Great Britain
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20
Estrogens and Related Substances in Plants, R. B. Bradbury and D. E. White, Department of Chemistry, University of Western Australia, Nedlands, Western Australia, pp. 207 to 230

Besonders relevant (Seite/Spalte/Zeile/Fig.):

21
Reproductive and General Metabolic effects of Phytoestrogens in Mammals, Rami S. Kaldas and Claude L. Hughes, Jr, Division of Reproductive Endocrinology and Infertility, Department of Obstetrics and Gynecology Duke Universitiy Medcal Center, Durham, North Carolina, Reproductive Toxicology Review Vo. 3, pp. 81-89, 1989, Printed in the USA
Besonders relevant (Seite/Spalte/Zeile/Fig.):

Fortsetzung auf Zusatzblatt [X]

B. **Sonstige Beweismittel**

Weitere Angaben auf Zusatzblatt [ ]

IX. Beweismittel

nur für EPA

Beigeschlossen = [X]

wird / werden nachgereicht = [ ]

A **Veröffentlichungen:** | Datum der Veröffentlichung

22
Determination of isoflavones in soy bean by high-perfomance liquid chromatography with amperometic detection, Yoshimi Kitada at all, Journal of Chromatography, 366 (1986) 403-406, Elsevier Science Publishers B. V. Amsterdamm – Printed in the Netherlands
Besonders relevant (Seite/Spalte/Zeile/Fig.):

23
Minireview, Flavones and isoflavones as inducing substances of legume nodulation, Barry G. Rolfe, BioFactors Vol. 1 no. 1 pp. 3 -10, 1988
Besonders relevant (Seite/Spalte/Zeile/Fig.):

Fortsetzung auf Zusatzblatt [ ]

B. **Sonstige Beweismittel**

Weitere Angaben auf Zusatzblatt [ ]

nur für EPA

X. Zahlung der Einspruchsgebühr erfolgt

☐ wie auf beigefügtem Gebührenzahlungsvordruck (EPA Form 1010) angegeben

☐

XI. Liste der Unterlagen:

Anlage Nr.:		Stückzahl:	
0	☒ Einspruchsformblatt	☐ (mind. 2)	
1	☒ Tatsachen und Begründung (s. VII.)	☐ (mind. 2)	
2	Kopien von als Beweismittel angegebenen (s. IX.)		
2a	☒ — Veröffentlichungen	23 (mind. je 2)	
2b	☐ — sonstigen Unterlagen	☐ (mind. je 2)	
3	☐ Unterzeichnete Vollmacht(en) (s. IV.)	☐	
4	☒ Gebührenzahlungsvordruck (s. X.)	1	
5	☒ Scheck	1	
6	☐ Zusatzblatt (Zusatzblätter)	☐ (mind. je 2)	
7	☐ Sonstige Unterlagen (bitte einzeln anführen):	☐	

XII. Unterschrift des Einsprechenden oder Vertreters

Ort Berlin

Datum 15.06.2005

Christlieb Klages
Rechtsanwalt

Name des (der) Unterzeichneten bitte mit Schreibmaschine wiederholen. Bei juristischen Personen bitte die Stellung des (der) Unterzeichneten innerhalb der Gesellschaft mit Schreibmaschine angeben

ZUR KASSE

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Berliner Volksbank

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nur zur Verrechnung

Europäisches Patentamt
Einspruch gegen EP0656786
Einsprechender: Jukunda
XI 766/05

Berlin
Ausstellungsort

15. 6. 2005
Datum

Unterschrift des Ausstellers

Der vorgedruckte Schecktext darf nicht geändert oder gestrichen werden. Die Angabe einer Zahlungsfrist auf dem Scheck gilt als nicht geschrieben.

Scheck-Nr.	X	Konto-Nr.	X	Betrag	X	Bankleitzahl	X	Text

000001102942S⑈ 5291489003⑈ 10090000⑈ 01⑈

Bitte dieses Feld nicht beschriften und nicht bestempeln

HERTIN
ANWALTSSOZIETÄT

HERTIN · Postfach 151460 · D-10676 Berlin

Europäisches Patentamt

80297 München

PROF DR. PAUL W. HERTIN
HERMANN-JOSEF OMSELS
DR. OLIVER SPIEKER
CHRISTLIEB KLAGES
Rechtsanwälte

DR. TOBIAS BOECKH
DR. SVEN LANGE
Patentanwälte
European Trademark Attorneys

Telefon: (030) 885 929-0
Telefax: (030) 885 92 929
Internet: www.hertin.de
Sekr.: Fr. Stein

Ihr Zeichen	Unser Zeichen	Berlin, den
EP 0 656 786	XI 766/05 ka	15.06.2005

In dem Einspruchsverfahren über das Europäische Patent

Veröffentlichungsnummer:	EP 0 656 786
Anmeldenummer:	93909679.8
Anmeldetag:	19.05.1993
Priorität:	AU 2511992 v. 19.05.1992
Titel:	Verwendung von Isoflavon Phyto-Östrogen Extrakten von Soja oder Klee.
Hinweis auf die Patenterteilung:	15.09.2004
Inhaber:	KELLY, Graham Edmund, Northbridge, NSW 2063 (AU)

beantragen wir,

1. den vollständigen Widerruf des gesamten Patents gemäß den Patentansprüchen 1 bis 11

2. hilfsweise mündliche Verhandlung

Zur

Begründung

führen wir folgendes aus:

Hiermit führen wir folgende Druckschriften in das Einspruchsverfahren ein:

E1: Conference Report: Consensus Development Converence: Prophylaxis and Treatment of Osteoporosis, January 1991 The American Journal of Medicine Volume 90: 107-110

E2: Commentary: The Role of Soy Products in Reducing Risk of Cancer, Mark Messina, Stephen Barnes, Journal of the National Cancer Institute, Vol. 83, No. 8, April 17, 1991: 541 - 546

E3: Naturally occurring oestrogens in foods – A review, K. R. Price and G. R. Fenwick, Food Additives and Contaminants, 1985, Vol. 2, No. 2, 73-106

E4: Use of a Mammalian Cell Culture Benzu(a)pyrene Metabolism Assay for the Detection of Potential Anticarcinogens from Natural Products: Inhibition of Metabolism by Biochanin A, an Isoflavone from Trifolium pretense L[1], John M. Cassady, Thomas M. Zennie, Young-Heum Chae, Mark A. Ferin, Nuris E. Portuondo, and William M. Baird, Cancer Research 48, 6257-6261, November 15, 1988

E5: Short Paper, Oestrogenic Activity of Soya-Bean Products, H. M. Drane, D. S. P. Patterson, B. A. Roberts and N. Saba, Central Veterinary Laboratory, Weybridge, Surry, KT15 3NB, England, Received 22 October 1979, Fd Cosmet, Toxicol. Vo. 18, pp 425-427

E6: Deutsche Tierärztliche Wochenschrift Nr. 11, Oestrogenwirksame Isoflavone in Trifolium pretense (Rotklee), Verteilung in den oberirdischen Pflanzenteilen und Vorkommen als "gebundene" Isoflavone, von G. Schultz – Mit 3 Abbildungen, aus dem Botanischen Institut der Tierärztlichen Hochschule Hannover, Direktor: Professor Dr. E. Perner: 246-251

E7: Nonsteroidal estrogenes of dietary origin: possible roles in hormone-dependent disease, KDR Setchell, SP Borriello, P Hulme, DN Kirk, and M Axelson, The American Journal of Clinical Nutrition 40: September 1984, pp 569-578. Printed in USA 1984 American Society for Clinical Nutrition

E8: Deutsche Tierärztliche Wochenschrift Nr. 17, Isoflavone in einigen Weiß- und Rotkleesorten und ihre oestrogene Wirksamkeit bei juvenilen Mäusen, von E. Grunert und Gudrun Woelke, aus der Klinik für Geburtshilfe und Gynäkologie des Rindes der Tierärztlichen Hochschule Hannover, Direktor: Prof. Dr. E. Aehnelt und G. Schultzs, aus dem Botanischen Institut der Tierärztlichen Hochschule Hannover – Direktor: Prof. Dr. E. Perner. 1. September, 431-433

E9: Soya – a dietary source of the non-steroidal oestrogen equol in man and animals. M. Axelson, J. Sjövall, B. E. Gustafsson and K. D. R. Setchell, Received 21 October 1983,

J. Endocr. (1984) 102, 49-56, 1984 Journal of Endocrinology Ltd, Printed in Great Britain, 0022-0795/84/0102-0049 $02.00/0

E10: Western diet and Western diseases: some hormonal and biochemical mechanismus and associations, Herman Adlercreutz, Department of Clinical Chemistry, University of Helsinki, Meilahti Hospital, Helsinki, Finnland, Scand J Clin Lab Invest 1190; 50, Suppl 201: 3-23

E11: Acta vet. scand. 1987, 28, 255-262. From the Department of Animal Hygiene, College of Veterinary Medicine, Helsinki and The Agricultural Research Centre, South Savo Research Station, Mikkeli, Finnland.: Variations in the Content of Plant Oestrogens in the Red Clover-Timothy-Grass during the Grwing Season. By K. Kallela, I. Saastamoinen and E. Huokuna. Acta vet. scand. vol. 28 no. 3-4 - 1987

E12: Sexual behaviour of ewes with clover disease treated repeatedly with oestradiol benzoate or testosterone propionate after ovariectomy, N. R. Adams, CSIRP Division of Animal Production, Private Bag, Wembley, Western Australia 6014, J. Reprod. Fert. (1983) 68. 113-117, Printed in Great Britain, 0022-4251/83/030113-05$02-00/0, 1983 Journals of Reproduction & Fertility Ltd.

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E16: Urinary excretion of lignangs and isoflavonoid phytoestrogens in Japanese men and women consuming a traditional Japanese diet. Herman Adelcreutz, Hideo Honjo, Akane Higashi, Theodore Fotsis, Esa Hämäläinen, Takeshi Hasegawa, and Hiroji Okada, Am J Clin Nutr 1991; 54:1093-1100. Printed in USA. 1991 American Society for Clinical Nutrition

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E18: Short Papers: Oestrogenic Response of the CD-1 Mouse to the Soya-Bean Isoflavones Genistein, Genistin and Daidzin, E. Farmakalidis and P. A. Murphy, Department of Food Technology, Iowa State University, Ames, IA 50011, USA, Received 1 October 1983, Fa Chem. Toxic. Vol. 22, no. 3, pp. 237-239, 1984, Printed in Great Britain

E19: Isoflavone Content of Soya-Based Laboratory Animal diets, P. A. Murphy, E. Farmakalidis and L. C. Johnson, Department of Food Technology, Iowa State University, Ames, I A 50011, USA, Received 4 August 1981, Fd Chem Toxic, Vol. 20. pp. 315 to 318, 1982, Printed in Great Britain

E20: Estrogens and Related Substances in Plants, R. B. Bradbury and D. E. White, Department of Chemistry, University of Western Australia, Nedlands, Western Australia, pp. 207 to 230

E21: Reproductive and General Metabolic effects of Phytoestrogens in Mammals, Rami S. Kaldas and Claude L. Hughes, Jr, Division of Reproductive Endocrinology and Infertility, Department of Obstetrics and Gynecology Duke Universitiy Medcal Center, Durham, North Carolina, Reproductive Toxicology Review Vo. 3, pp. 81-89, 1989, Printed in the USA

E22 Determination of isoflavones in soy bean by high-perfomance liquid chromatography with amperometic detection, Yoshimi Kitada at all, Journal of Chromatography, 366 (1986) 403-406, Elsevier Science Publishers B. V. Amsterdamm – Printed in the Netherlands

E23 Minireview, Flavones and isoflavones as inducing substances of legume nodulation, Barry G. Rolfe, BioFactors Vol. 1 no. 1 pp. 3 -10, 1988

Die Druckschriften sind vor dem Prioritätstag des Streitpatents EP 0 656 786 veröffentlicht worden und damit Stand der Technik nach Art. 54 (2) EPÜ. Gutachterlich wird weiterhin auf die Dokumente verwiesen, die im Anmeldeverfahren berücksichtigt wurden. Hierbei handelt es sich um die Dokumente D1 bis D33, die in der Anlage A mit Schreiben vom 21. Juli 2003 von den Vertretern des Anmelders gegenüber dem Europäischen Patentamt aufgelistet wurden.

I. Vorbemerkung

I.1. Begriffsklärung

Gemäß Hauptanspruch des o. g. Streitpatentes wird die Verwendung eines Isoflavon-Phytoöstrogen-Extraktes von Soja oder Klee beansprucht, wobei dieser Extrakt in Form von Dosierungseinheitsformen für die Behandlung des prämenstruellen Syndroms, von Symptomen, die mit der Menopause verbunden sind, oder von Prostatakrebs verwendet wird. Der in Anspruch 1 verwendete Begriff der Dosierungseinheitsform ist nicht selbsterklärend und wird in dem o. g. Streitpatent nicht weiter erläutert. Daher soll im folgenden davon ausgegangen werden, dass dieses technische Merkmal allgemein bekannte galenische Formulierungen betrifft. Die technischen Merkmale prämenstruelles Syndrom und Symptome, die mit der Menopause verbunden sind, sollen im folgenden so verstanden werden, dass es sich um Abweichungen von dem durchschnittlichen Gesundheitszustand handelt, die natürlicher Weise im Zusammenhang mit dem Regelzyklus bzw. im Zusammenhang mit dem Wechseljahren der Frauen auftreten.

II. Syndrome und Symptome sind keine Krankheiten im Sinne der Rechtsprechung der Beschwerdekammern des EPA

Der technische Charakter sowie die gewerbliche Anwendbarkeit bei der zweiten medizinischen Indikation ist nur gegeben, wenn die zu behandelnden Krankheiten konkret im Anspruch genannt werden. Die Begriffe prämenstruelles Syndrom und Symptome, die mit der Menopause verbunden sind, sind derartig vage, dass keine konkret zu behandelnde Krankheit offenbart ist. Das Patent ist daher wegen Nichtpatentierbarkeit gemäß Art. 52 (4) zu widerrufen.

III. Vorbemerkung zum Stand der Technik vor dem Prioritätsdatum – eine Replik auf die Darstellung des Standes der Technik seitens des Anmelders mit Schreiben vom 21. Juli 2003 (Seite 3)

Gemäß den Ausführungen vom 21. Juli 2003 der Vertreter des Anmelders im Patentanmeldeverfahren zu o. g. Streitpatent sei der Fachmann vor dem Prioritätstag der o. g. Patentanmeldung davon ausgegangen, dass Isoflavone in Form Phytoöstrogenen nachteilhafte Effekte bei der Anwendung in Mensch oder Tier zeigen. Hierbei verweist er auf das Dokument D32, welches diesseitig als E21 ins Verfahren eingeführt wurde. Gemäß den Ausführungen des Anmelders bewegt sich die technische Lehre o. g. Patentanmeldung vom Stand der Technik weg. Hierzu ist zweierlei anzumerken; 1.: Ein einziges Dokumente kann nicht herangezogen werden, um den Stand der Technik vor einem bestimmten Datum darzustellen bzw. um die allgemeine Sichtweisen der Fachwelt zu erläutern. 2.: Die Darstellung des Inhalts von E21 / D32 ist inhaltlich nicht korrekt. Bei der genannten Einzelpublikation handelt es sich um einen Review, der im Journal für Reproductive Toxicology erschienen ist. In diesem Artikel wird dargestellt, dass Phytoöstrogene (Seite 1) beispielsweise aus Klee oder Soja (Tabelle 1) verschiedene Wirkungen im humanen Bereich haben. Diese Wirkungen hängen selbstverständlich von der Dosis der eingesetzten Phytoöstrogene (zu denen auch Isoflavone gehören) ab. So wird beispielsweise auf Seite 88 ausgeführt, dass Phytoöstrogene einen heilenden Effekt bei Krebs haben aber auch bei Symptomen, die mit der Menopause bei Frauen im Zusammenhang stehen (rechts Spalte, vorletzter Absatz). Es ist selbstverständlich, dass Östrogene / Phytoöstrogene (mit einem ihrer Hauptbestandteile Isoflavon) nicht in jeder Konzentration bei Frauen jeden Lebensalters (Pubertät bzw. nach den Wechseljahren) eingesetzt werden können. Insbesondere im Bereich der reproduktiven Medizin (dem Schwerpunkt oben genannter

Zeitschrift) können natürlich nicht beliebige Dosen von Östrogenen / Phytoöstrogenen eingenommen werden, da ab einer bestimmten Dosis von Östrogenen / Phytoöstrogenen Unfruchtbarkeit auftritt. E21 offenbart in Abhängigkeit von der Dosis positive Wirkungen von Phytoöstrogenen bei Krebs sowie bei menopausalen Symptomen und führt daher keines falls vom Gegenstand der Erfindung weg.

IV. Hauptargumentationen des Anmelders in Bezug auf die im Anmeldeverfahren entgegengehaltenen Dokumente D1 bis D33

Im Schreiben vom 21. Juli 2003 setzt sich der Anmelder mit den im Prüfungsverfahren entgegengehaltenen Druckschriften auseinander. Im Bezug auf die Dokumente D4 (JP-A-62-106016), D5 (EP 0 129 667), D14 (Adlerkreutz (1992) Lancet 339:1223) führt er aus, dass in diesen Dokumenten die Verwendung von Phytoöstrogenen und damit von Isoflavonen für die beanspruchten Krankheiten und Symptomen offenbart ist. Aus den genannten Publikationen würde aber nicht hervorgehen, dass die Phytoöstrogene / Isoflavone aus Soja oder Klee stammen. Gemäß den Ausführungen des Anmelders scheint es also die besondere erfinderische Leistung zu sein, die an sich bekannten Isoflavon–Phytoöstrogen-Extrakte zur Behandlung der genannten Krankheiten aus Soja oder Klee zu gewinnen. Die Aufnahme dieses technischen Merkmals war gemäß den Unterlagen des Anmeldeverfahrens entscheidend für die Erteilung o. g. Streitpatentes. Im Folgenden wird daher bei der Erörterung der einzelnen Ansprüche immer auch zu erörtern sein, inwieweit es sich bei den technischen Merkmal „von Soja oder Klee" um ein Merkmal handelt, welches einen erfinderischen Schritt widerspiegelt.

V. Die fehlende Neuheit bzw. erfinderische Tätigkeit der Ansprüche des Streitpatentes

Anspruch 1

Der verwendete Isoflavon-Phytoöstrogen-Extrakt im Sinne der Erfindung scheint ein Pflanzenextrakt aus Soja oder Klee zu sein der das Phytoöstrogen Isoflavon enthält. Isoflavone sind bekannte Bestandteile von phytoöstrogenen Extrakten (E6 S. 1, E13 S. 1, E15 S. 155, E16 Tabelle 5, E17 Tabelle 1 und Figur 1, E18 S. 237, E19 S. 315).

E21 offenbart auf Seite 88 die Verwendung von phytoöstrogenen Extrakten zur Behandlung von Krebs bzw. von Symptomen von Frauen in der Menopause, d. h. von Symptomen, die mit der Menopause verbunden sind.

E16 verweist speziell auf den Zusammenhang zwischen Isoflavonen und Prostatakrebs (S. 1093). Weiterhin beschreibt E16 im Zusammenhang von Isoflavonen-Phytoöstrogenen-Extrakten bei pre- und postmenopausalen Frauen (S. 1096) und den damit auftretenden Symptomen.

Die antikanzerogene Wirkung dieser Phytoöstrogen-Extrakte insbesondere bei hormonabhängigen Krebsen wird auch in E14 diskutiert (S. 791 und S. 795 bis 796).

E10 verweist ebenfalls auf die hormonabhängigen Krebse insbesondere Prostatakrebs (Zusammenfassung sowie S. 3, S. 4 linke Spalte, S. 10), insbesondere wird auf Sojaprodukte hingewiesen (S. 8).

E9 offenbart auf S. 54 (rechte Spalte sowie auf S. 55) verschiedene Phytoöstrogene aus Soja, die einen Effekt auf Menstruationsbeschwerden haben. Insbesondere wird in E9 auch noch einmal auf die hormonabhängigen Tumoren verwiesen, zu denen o. g. Entgegenhaltungen insbesondere der Prostatakrebs zählt.

Weiterhin werden in E7 Wirkungen von phytoöstrogen Extrakten, die auch immer Isoflavone enthalten, im Zusammenhang mit Menstruationsbeschwerden diskutiert (S. 750 sowie S. 574, rechte Spalte).

E4 beschreibt die Wirkung von Kleeextrakten (S. 6257), die Isoflavone umfassen, auf Tumoren.

In E2 wird die Wirkung von Extrakten aus Soja, die Phytoöstrogene und somit Isoflavone umfassen (siehe S. 545 rechte Spalte), auf Frauen mit Menstruationsbeschwerden offenbart (S. 542).

Jede dieser einzelnen Druckschriften offenbart daher zumindest die Verwendung von phytoöstrogen Extrakten, die immer Isoflavon umfassende Extrakte und somit Isoflavon-Phytoöstrogen-Extrakte sind, im Zusammenhang mit den in Anspruch 1 alternativ aufgezählten Syndromen, Symptomen oder Prostatakrebs. Die Erfindung wird daher durch diese Entgegenhaltungen nahe gelegt. Gemäß den Ausführungen seines Schreibens vom 21. Juli 2003 scheint auch der Anmelder / Patentinhaber o. g. Streitpatentes davon auszugehen, dass im Stand der Technik entsprechende Offenbarungen zu finden sind. Der so genannte „entgegenstehenden Blick der Fachwelt" (siehe Punkt III) ist bereits diskutiert worden und muss angesichts der Vielzahl der dieser Argumentation widersprechenden Entgegenhaltungen nicht weiter erörtert werden. Die Offenbarung der genannten Druckschriften unterscheidet sich vom

Hauptanspruch o. g. Patentanmeldung dadurch, dass nicht bei jeder Offenbarung explizit darauf hingewiesen wird, dass die Isoflavon-Phytoöstrogen-Extrakte aus Soja oder Klee gewonnen worden sind.

Einzelne Entgegenhaltungen wie z. B. E9 weisen aber auf Sojaprodukte hin. In E4 wird als Quelle der Phytoöstrogene Kleeextrakt genannt. Auch E10 nennt als eine Quelle der Phytoöstrogene Klee (S. 9); auch E14 verweist auf Klee (S. 793) und E19 benennt als Quelle der isoflavonoiden Östrogene Sojaprodukte (S. 1093). Denn diese genannten Druckschriften sind daher für einzelne Alternativen, d. h. Soja oder Klee für die alternativ aufgezählten Syndrome, Symptome bzw. für den hormon-abhängigen Prostatakrebs. Die Druckschriften, die nicht neuheitsschädlich für einzelne Alternativen des Anspruchs 1 sind, legen die Erfindung nahe, da sie den Zusammenhang von Phytoöstrogenen und Menstruationsbeschwerden oder Prostatakrebs offenbaren, ohne als Quelle für die Phytoöstrogene Klee oder Soja zu benennen. Es ist jedoch dem Fachmann seit 1931 bzw. 1946 bekannt (E5, S. 425 und E6 S. 1), dass Klee phytoöstrogene Bestandteile bzw. Extrakte enthält, die auch immer Isoflavone umfassen. Auch E3 weist darauf hin, dass der Zusammenhang von Phytoöstrogenen aus Pflanzen, insbesondere aus Sojapflanzen (Tabelle 3) bzw. Klee (Tabelle 1) bei Unterleibsproblemen von Frauen seit über 2.000 Jahren bekannt ist. Gemäß E3 sind seit 1975 300 Pflanzen bekannt, die Phytoöstrogene (Isoflavone) aufweisen (S. 73).

Das prämenstruelle Syndrom bzw. Symptome, die mit der Menopause verbunden sind haben ihre Ursache in zu hohen oder zu niedrigen Dosen an Östrogenen. Daher ist es selbstverständlich, dass man in der Vergangenheit versucht hat, derartige Probleme mit einer Hormonersatztherapie zu minimieren. Phytoöstrogene können die Wirkung der fehlenden Östrogene substituieren. Auf die Dosisabhängigkeit der Gabe von Phytoöstrogenen verweist E3 (Tabelle 6) aber auch alle Entgegenhaltungen, die die Kleekrankheit bei Schafen in Australien erwähnen (z. B. E6 oder E8), bei denen die erhöhte Aufnahme von Phytoöstrogenen zu menopausalen Problemen führt. Daher ist es nicht verwunderlich, dass bestimmte Entgegenhaltungen, wie z. B. E21 darauf verweisen, dass bei einer erhöhten Gabe von Phytoöstrogenen bestimmte Probleme auftreten können. So wäre es beispielsweise nicht vorteilhaft, wenn Isoflavon-Phytoöstrogen-Extrakte von sehr jungen Schafen oder Ziegen bzw. von sehr jungen Frauen aufgenommen werden, da hier Probleme der Unfruchtbarkeit auftreten können. Derartige Offenbarungen führen den Fachmann jedoch nicht von der erfindungsgemäßen Lehre weg, sondern motivieren ihn, derartige Extrakte entweder nur bei Frauen bzw. Tieren in bestimmten Lebensabschnitten – beispielsweise nach den Wechseljahren – oder in einer bestimmten do-

sierten Form zu geben, wobei die Dosierung – nach Tabelle 6 von E3 – davon abhängt ob eine postmenopausale Therapie oder eine Geburtenkontrolle mit Hilfe der Isoflavon-Phytoöstrogen-Extrakte durchgeführt werden soll.

Angesichts des Jahrzehnte alten Wissens (siehe E6 und E5 oder E3) über den Zusammenhang der Aufnahme von Isoflavon-Phytoöstrogen-Extrakten von Klee die mit bestimmten Phänomenen in Tieren oder Menschen assoziiert sind, die mit der Menopause oder der Fruchtbarkeit verbunden sind, es zu fragen, ob bei dem o. g. Streitpatent nicht ähnlich wie bei den sog. Niembaum-Patent jahrelanges Wissen einer Bevölkerungsgruppe (hier australische Schafzüchter) von einem australischen Anmelder im nachhinein durch ein Patent monopolisiert werden soll.

Anspruch 2

Dieser Anspruch ist abhängig von Anspruch 1, so dass auf obige Ausführungen verwiesen kann. Anspruch 2 betrifft eine Verwendung, die dadurch gekennzeichnet ist, dass das Medikament ein nahrungsmittelgeeignetes Excipiens umfasst. Es ist eine platte Selbstverständlichkeit, dass Medikamente verschiedene Trägerstoffe umfassen müssen. Aus diesem Grunde gelten die gleichen Entgegenhaltungen, die für Anspruch 1 relevant sind auch für Anspruch 2, d. h. Anspruch 2 wird durch die in Anspruch 1 genannten Entgegenhaltungen nahe gelegt, da dieser Anspruch kein erfindungswesentlichen Gegenstand aufweist.

Anspruch 3

Anspruch 3 bezieht sich auf Anspruch 1 bzw. 2, wobei das Isoflavon-Phytoöstrogen (gemeint ist vermutlich der Isoflavon-Phytoöstrogen-Extrakt) aus Soja extrahiert wird. In Ausführungen zu Anspruch 1 sind zahlreiche Entgegenhaltungen genannt worden, aus denen hervorgeht, dass Isoflavon als Phytoöstrogen aus Soja extrahiert werden kann. So offenbart z. B. E2 verschiedene Extraktionsverfahren für die Auftrennung von Isoflavonen aus Soja (siehe auch Abbildungen 2, 3 und 4 von E7; bzw. Figur 5 von E9; Figur 2 und 3 von E17). Anspruch 3 ist daher nicht erfinderisch im Hinblick auf die genannten Entgegenhaltungen.

Anspruch 4

Anspruch 4 konkretisiert Anspruch 3 dahingehend, dass das Isoflavon-Phytoöstrogen aus Sojahypokotylen extrahiert wird. Dem Fachmann ist bekannt, dass er aus sämtlichen Soja- bzw. Kleepflanzenbestandteilen Isoflavone gewinnen kann. Da in der Anmeldung keine besonderen Vorteile für die Gewinnung aus den Hypokotyl offenbart werden, ist dieser Bestandteil der Pflanze scheinbar genauso gut geeignet wie sämtliche andere Bestandteile auch. Anspruch 4 ist daher nicht erfinderisch.

Anspruch 5

In Anspruch 5 wird die Verwendung gemäß Anspruch 1 oder 2 dahingehend konkretisiert, dass das Isoflavon-Phytoöstrogen aus Klee extrahiert wird. Es ist aus E6 (S. 1) bekannt, dass Isoflavone aus Klee gewonnen werden können.

Weiterhin führt E3 (Tabelle 1) die Gewinnung von Isoflavonen aus Klee aus. In E20 (Tabelle 1, S. 213) wird ebenfalls die Gewinnung von Isoflavonen aus Klee offenbart. Variationen von einzelnen Isoflavon-Phytoöstrogenen aus Klee werden auch in E11 (S. 257) beschrieben. Demgemäß ist der Anspruch 5 im Lichte jeder der genannten Entgegenhaltungen nicht erfinderisch.

Anspruch 6

Anspruch 6 konkretisiert die chemischen Vertreter, die den Isoflavon-Phytoöstrogen-Extrakt bilden. Hierbei handelt es sich um Genistein, Daidzein oder Glycoside davon. Anspruch 6 ist von den Ansprüchen 1 bis 4 abhängig, so dass auch die oben genannten Ausführungen verwiesen werden kann. Dem Fachmann ist bekannt, dass Isoflavon-Phytoöstrogen-Extrakte die in Anspruch 6 genannten chemischen Vertreter umfassen. So wird beispielsweise in E22 (Figur 7) auf diese Komponenten aus Soja verwiesen und E11 zeigt auf S. 257 die beanspruchten chemischen Vertreter aus Klee. E4 offenbart in Figur 3 die genannten chemischen Vertreter. Aus diesem Grunde ist Anspruch 6 nicht erfinderisch.

Anspruch 7

Anspruch 7 betrifft die Verwendung gemäß Anspruch 1 oder 2, wobei der Isoflavon-Phytoöstrogen-Extrakt Genistein und/oder Biochanin A: Daidzein und/oder Formonoetin umfasst, die in einem Verhältnis von ungefähr 1:2 bis 2:1 vorhanden sind. Für die Auswahl dieses Bereiches werden in o. g. Streitpatent keine besonderen, überraschenden Effekte gezeigt, so dass diese Bereichsangabe willkürlich ist. Anspruch 7 ist daher nicht erfinderisch im Lichte der Entgegenhaltungen die sie für Anspruch 1, 2 aber auch 6 genannt werden. Weiterhin zeigen die spektroskopischen Analysen in E11 auf S. 257 sowie in E22 in Figur 2, 4 und 6, dass es sich bei diesen genannten Bereichen um die natürlicherweise auftretenden Bereiche der genannten chemischen Vertreter in Isoflavon-Phytoöstrogen-Extrakten handelt. Auch E3 zeigt in Tabelle 3 ähnliche Verhältnisse, wie sie im Streitpatent beansprucht werden. Die in E3 genannten Verhältnisse entsprechen wie auch in E11 den natürlicherweise vorkommenden Isoflavonen in Soja und dessen Produkten, wie sie in Anspruch 7 beansprucht werden.

Anspruch 8

Anspruch 8 ist abhängig von allen vorangegangenen Ansprüchen und konkretisiert die Dosiseinheit der Medikamente auf den Bereich von 20 bis 200 mg bzw. von 50 bis 150 mg. Auch für diese Bereichsangabe werden im Streitpatent keine überraschenden Effekte gezeigt, so dass diese Bereichsauswahl willkürlich ist und keinen erfinderischen Schritt begründen kann. Anspruch 8 ist daher nicht erfinderisch im Licht der genannten Entgegenhaltungen. In E8 werden verschiedene Bereichsangaben der Applikation von Isoflavonen angegeben (so beispielsweise in Tabelle 4 aber auch auf S. 33, die letzten beiden Abschnitte). Diese Konzentration der Phytoöstrogene entspricht ungefähr der Menge der Dosiseinheiten wie in Anspruch 8 beansprucht; im Hinblick auf diese Entgegenhaltung sowie auf den allgemeinen Stand der Technik ist dieser Anspruch wie bereits oben ausgeführt nicht erfinderisch.

Anspruch 9

Bezüglich der Auswahl des Bereichs von über einem Monat gelten die gleichen Ausführungen wie zu Anspruch 7 oder 8, nämlich, dass für diese Bereichsangabe keine besonderen Effekte gezeigt werden können; sie sind daher willkürlich und können keinen erfinderischen Schritt begründen.

Anspruch 10

Anspruch 10 ist nicht erfinderisch, da die genannten Verbindungen Coumestane, Lignane und Flavone natürlicherweise in Soja bzw. Kleeextrakten vorkommen. Diese beanspruchten Verbindungen sind gemäß Figur 2 in E3 sowie gemäß Figur 3, Tabelle 2 sowie S. 85 letzter Abschnitt häufig zu findende Phytoöstrogene bzw. Verbindung die mit diesen assoziiert auftreten (so auch E10 S. 11 linkte Spalte, S. 13 rechte Spalte, S. 7 rechte Spalte; E16 S. 1097 linke Spalte und E23 Figur 2). Diese genannten Verbindungen werden keine besonderen Effekte offenbart, so dass Anspruch 10 die erfinderische Tätigkeit fehlt.

Anspruch 11

Anspruch 11 bezieht sich die vorangegangenen Ansprüche, wobei die Dosierungseinheitsform die eigentlich nur in Anspruch 1 offenbart ist eine Tablette oder Kapsel ist. Tabletten oder Kapseln gehören zu den üblichen galenischen Zubereitungsformen, für die in der Anmeldung keine besonderen überraschenden Effekte gezeigt werden können. Der Anspruch ist daher nicht erfinderisch im Lichte des allgemeinen Fachwissens.

Mithin ist der Einspruch begründet.

Beglaubigte und einfache Abschrift anbei.

i.V. Klages
Rechtsanwalt

CONFERENCE REPORT

Consensus Development Conference: Prophylaxis and Treatment of Osteoporosis

A consensus development conference sponsored by the National Institute of Arthritis and Musculoskeletal and Skin Diseases, the European Foundation for Osteoporosis and Bone Disease, and the American National Osteoporosis Foundation was held in Copenhagen October 19 and 20, 1990. A panel of 14 listened to evidence from experts in a public session attended by 2,000 people, including representatives of the medical profession, the pharmaceutical industry, the press, and ministries of health. After a closed session, the panel discussed its report with the audience and a consensus statement was then presented at a press conference.

DEFINITION

Osteoporosis is a disease characterized by low bone mass, microarchitectural deterioration of bone tissue leading to enhanced bone fragility, and a consequent increase in fracture risk.

SIGNIFICANCE

Osteoporosis is a major cause of mortality, morbidity, and medical expense worldwide. It affects an estimated 75 million people in the United States, Europe, and Japan combined, including one in three postmenopausal women and a majority of the elderly. Osteoporosis causes more than 1,300,000 fractures annually in the United States alone. The disease will be an even greater problem in the future, because the world population is aging and the incidence of osteoporotic fractures is increasing in many geographic areas.

Hip fracture is responsible for much of the mortality and morbidity of osteoporosis and is a leading cause of disability in the aged. Twelve percent to 20% of patients with hip fracture will die within 1 year of the event, and mortality rises progressively with advancing age. Moreover, the majority of hip fracture survivors are unable to perform the activities of daily living unaided. A small but significant percentage will require permanent custodial and nursing care.

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The high frequency of falls with advancing age contributes significantly to the likelihood of hip and other fractures in the elderly. Spine fractures are common causes of pain, deformity, loss of height, and disability. The financial cost of osteoporosis in the United States each year exceeds 10 billion dollars.

RISK FACTORS AND CAUSES

Bone mass in the elderly reflects the accumulation and maintenance of bone tissue during growth and maturation, and the rate and duration of bone loss thereafter. Factors that predispose to osteoporosis are those inducing a low peak bone mass and those that underlie excessive postmenopausal and aging-associated bone loss. Genetic, endocrine and lifestyle factors are contributory, although our understanding of these factors is incomplete.

White and Asian women who are thin or petite and have a family history of the disease are at greatest risk for osteoporosis. Estrogen deficiency is the main cause of rapid postmenopausal bone loss and contributes to aging-associated losses as well. Consequently, an early menopause may hasten the appearance of future osteoporosis. Premenopausal estrogen deficiency states also promote bone loss. Prolonged periods of inadequate calcium nutrition increase risk, and cigarette smoking, alcohol abuse, and a sedentary lifestyle are suspected risk factors.

Diseases or conditions known to cause osteoporosis (secondary osteoporosis) include excessive exposure to glucocorticoids, hyperthyroidism, primary hyperparathyroidism, immobilization, and multiple myeloma. Although white men and blacks of both sexes are at lowest risk, osteoporosis appears in all populations, and one in five hip fractures occurs in men.

PREVENTION AND TREATMENT

Inhibitors of Bone Resorption

ESTROGENS: Osteoporosis can be prevented. Estrogen therapy is the drug of choice for preventing bone loss in women after the menopause or in women with impaired ovarian function. Estrogen, by inhibiting bone resorption, reduces bone loss at all skeletal sites. The effects of estrogen persist for as long as therapy continues. The minimum fully effective oral doses are 0.625 mg conjugated equine estrogen or piperazine estrone sulphate per day; 2

mg 17β-estradiol per day; and 50 to 100 μg transdermal estradiol per day.

Epidemiologic data suggest that estrogen therapy given for at least 5 years early in the climacteric period reduces subsequent hip and Colles' fractures by about 50% and vertebral fractures by up to 90%.

The addition of a progestagen does not appear to impair the response of the skeleton to estrogen; some progestational agents may enhance the effect of estrogen. Estrogens may also be used in the treatment of established osteoporosis. The positive effects of estrogen have been demonstrated in patients up to the age of 70 years.

Critical review of published observational studies suggests that estrogen therapy decreases the risk of cardiovascular disease by about 50%, with a similar effect on overall mortality. It is not known whether combined therapy with progestagen modifies this benefit.

There is no consistent increase in the risk of breast cancer among women who have ever used estrogen replacement therapy. Long-term therapy (more than 10 years) may be associated with a small increase in the *diagnosis* of breast cancer, but no increase in the risk of breast cancer *death* has been shown. There is inadequate evidence to assess the effects of combined therapy on the risk of breast cancer.

Use of unopposed estrogen therapy increases the risk of endometrial cancer; adequate doses of a progestagen negate this increase.

CALCITONIN: Calcitonin decreases further bone loss at vertebral and femoral sites in established osteoporosis, but effects on fracture frequency have not been published. Calcitonin may exert an analgesic effect in patients with acute bone pain due to vertebral fractures. Therapeutic regimens include injections or nasal spray. The effect of calcitonin is greater in patients with high-turnover osteoporosis. In some studies, calcitonin has been shown to prevent trabecular bone loss during the first years of menopause. It is not established whether there is an effect on cortical bone. Calcitonin appears to inhibit further bone loss in glucocorticoid-induced osteoporosis. The efficacy of calcitonin has not been determined in osteoporosis in men. Calcitonin provides an alternative in prevention of menopause-related bone loss in those women who are unable or unwilling to take estrogen.

BISPHOSPHONATES: Several well-controlled studies indicate that orally administered bisphosphonates reduce bone loss and the incidence of vertebral deformity in patients with established postmenopausal osteoporosis. Data suggest that bisphosphonates reduce bone loss during the first years of the menopause and in patients with glucocorticoid-mediated osteoporosis. Effects on non-vertebral fracture and long-term skeletal impact remain to be assessed.

CALCIUM: The importance of an adequate calcium intake at all stages of life is well established. It is a prerequisite for normal bone growth and for the attainment of peak bone mass. However, a high calcium intake will not substitute for estrogen therapy in blunting the accelerated bone loss during the climacteric period. Maintenance of an adequate calcium intake is also necessary in elderly subjects. A minimum intake of 800 mg of calcium daily is recommended for all adults. Higher amounts are required in childhood, adolescence, pregnancy, lactation, and old age.

Stimulators of Bone Formation

The ideal therapy for established osteoporosis should stimulate bone formation and increase bone mass sufficiently to decrease the occurrence of new fractures.

FLUORIDE: Fluoride stimulates osteoblasts and increases cancellous bone mass. However, its effect on the incidence of fracture is controversial and might be dose dependent. In a recent controlled clinical trial that assessed the efficacy of 75 mg of sodium fluoride per day, trabecular bone mass of the spine was increased, but the incidence of vertebral fracture was unchanged, and non-vertebral fractures, including complete and incomplete, were increased. Earlier, less well-controlled studies at lower fluoride dosages (up to 50 mg of sodium fluoride) showed a beneficial effect on the vertebral fracture rate in both postmenopausal and glucocorticoid-induced osteoporosis. This might imply a narrow therapeutic window for fluoride and deserves further study. The known side effects of fluoride—gastrointestinal effects and arthralgias—have been decreased by changing the formulation, using other fluoride salts, and reducing the dose, but still remain a problem.

ANABOLIC STEROIDS: Currently available anabolic steroids can increase bone mass in osteoporosis, perhaps by increasing bone formation. Their long-term use is limited by side effects, which may include virilization as well as adverse effects on carbohydrate and lipid metabolism and on liver function.

PARATHYROID HORMONE: Promising data, in which parathyroid hormone increases total bone mass, justify the continued clinical investigation of this hormone as an anabolic treatment.

OTHER MODALITIES: The positive effect of 1,25-dihydroxyvitamin D_3 and 1α-hydroxyvitamin D on the incidence of fracture disclosed in some studies

in osteoporotic subjects may reflect promotion of calcium absorption, especially in the elderly and those on low calcium intakes.

In conjunction with calcium intake, weight-bearing exercise contributes to the development and maintenance of bone mass. In contrast, excessive exercise sufficient to cause amenorrhea is associated with bone loss due to accompanying ovarian insufficiency. There is yet no evidence that moderate physical exercise retards bone loss associated with menopause or aging. However, active exercise is considered useful in the elderly, particularly in improving muscular function and agility and in reducing the likelihood of falls.

Other Approaches to Fracture Prevention

Falling is the precipitating event in the majority of osteoporosis-related fractures in the elderly. Reductions in balance, muscle strength, and agility caused by aging, medications, and diseases are contributory. Hence, every effort must be made to reduce environmental hazards and modify therapeutic regimens that may predispose to falling. Vitamin D deficiency, which is commonplace in housebound elderly and may magnify the risk of hip fracture, should be prevented and treated.

SELECTION FOR EARLY AND LATE INTERVENTION

Bone Mass Measurement: Methodology

There are four basic techniques currently used for non-invasive assessment of the skeleton: single photon absorptiometry (SPA), dual photon absorptiometry (DPA), dual energy x-ray absorptiometry (DXA), and quantitative computed tomography (QCT). Other techniques, such as neutron activation analysis, compton scattering, ultrasonic transmission, and magnetic resonance either are not widely applicable or are incompletely developed.

SPA incorporates an isotope source, iodine 125, and a scintillation detector, and can be used to assess bone mass in the peripheral skeleton at sites where cortical bone predominates, such as the midshaft of the radius. Technical advances in SPA have improved both the precision and sensitivity of the method, and have provided the capability to assess trabecular bone-enriched sites, such as the distal end of the radius or the calcaneus.

DPA incorporates a dual-energy isotope, gadolinium 153, which permits scanning of thicker body parts. While spine and hip assessment procedures are most widely used, DPA may be employed for the quantification of total body bone mass or any designated segment thereof. It is a somewhat time-consuming procedure.

DXA utilizes an x-ray source in place of an isotope source and provides improvements in detector configuration and automation of analysis procedures. These have greatly increased both the speed and the precision of bone mass measurements. Overall performance has thereby been enhanced. Moreover, newer applications, such as lateral spine scanning in DXA, have been developed.

QCT allows the direct measurement of trabecular or total bone density, principally in the spine. Recent advances in software and hardware have substantially improved QCT performance by controlling technical parameters and automating the procedure. Decreases in scanning time, reductions in the radiation dose, which still remains high, and semi-automated analyses have enhanced the clinical utility of this technique.

Bone Mass Measurements in Diagnosis and Assessment of Therapy

Three general clinical needs can be addressed with bone mass measurements: (1) assessment of asymptomatic individuals, particularly perimenopausal women, to determine whether intervention is indicated on the basis of increased fracture risk; (2) diagnosis of osteoporosis in patients with symptoms or with other clinical indications, such as suspected low bone mass on radiographs; and (3) monitoring of treatment efficacy.

Each of these clinical applications places different requirements upon the techniques employed for bone mass measurement.

For purposes of assessment, the technique must be simple, safe, and inexpensive, and it should relate adequately to the patient's overall fracture risk (irrespective of fracture site). Bone mass measurements are required for accurate assessment of fracture risk in the individual patient. It is now established that a single accurate measurement of bone mass at any site has equal predictive value for subsequent fractures of all types. The diagnosis of osteoporosis affecting a particular site of the skeleton requires bone mass assessment at the appropriate site. For example, if vertebral deformities are detected on radiographs, measurement of spinal bone mass can determine whether significant spinal osteoporosis is present. Monitoring of treatment efficacy requires measurement techniques with high precision. In addition, the measurement site and its cortical/trabecular composition must adequately reflect the effects of particular therapeutic agents.

In addition to levels of bone mass, clinical decision-making also requires consideration of other factors such as current age, life expectancy, and anticipated (or measured) rate of bone loss (see be-

low). Models have now been proposed that incorporate these variables and provide estimates of remaining lifetime fracture probability. Such models can assist in determining which patients will benefit most from specific interventions.

Biochemical Assessment of Bone Remodeling and Osteoporosis

In the diagnosis of osteoporosis, it is necessary to exclude osteomalacia and to assess secondary causes of osteoporosis. Biochemical markers of bone turnover are a routine part of the diagnostic examination of patients with a number of metabolic bone diseases. They are also useful in the follow-up of such patients, e.g., to monitor the effects of intervention.

Markers of bone formation include serum total or bone-specific alkaline phosphatase activity, serum osteocalcin (bone gla-protein), and the serum level of type I collagen propeptides.

Osteocalcin, the only protein that is specific for bone, is a sensitive and specific marker of osteoblastic activity. Radioimmunoassays of the carboxy- and amino-terminal propeptides of type I collagen have been developed; their sensitivity and specificity require further investigation.

Markers of bone resorption most commonly used are urinary calcium and hydroxyproline. Recently, efforts have been made to develop even more sensitive markers of bone resorption. Pyridinoline and deoxy-pyridinoline, derived from two crosslinks of collagen that appear to be specific for bone and cartilage collagen, have become available recently. Their levels are elevated in diseases characterized by a high bone turnover, are increased after the menopause, correlate with bone resorption measured on iliac crest biopsy specimens, and are not influenced by diet. Plasma tartrate-resistant acid phosphatase reflects osteoclast activity but improved assays are needed.

There is growing evidence that the rate of postmenopausal bone loss can be determined by biochemical markers. One examination shortly after the menopause may help to predict the degree of bone loss in conjunction with a measurement of bone mass.

URGENT NEED FOR RESEARCH

Research is urgently needed in the following areas: (1) in enhancing understanding of the epidemiology of osteoporosis; (2) in improving our understanding of the regulation of bone remodeling and mass, including nutritional, mechanical, endocrine, and local factors; (3) in seeking new approaches to reduce postmenopausal and aging-associated bone loss; (4) in determining the factors that affect the accumulation of bone tissue during growth and maturation; (5) in identifying effective, safe, and inexpensive methods to restore healthy bone in osteoporotic patients; (6) in discovering accurate, easily accessible, and inexpensive methods to predict the likelihood of osteoporosis and to monitor the response to therapy; and (7) in expanding our knowledge of falling among the elderly (risk factors, causes), in order to design effective prevention approaches.

CONFERENCE PARTICIPANTS

The following individuals were on the panel: R. Bouillon, Belgium; P. Burckhardt, Switzerland; C. Christiansen, Denmark; H.A. Fleisch, Switzerland; T. Fujita, Japan; C. Gennari, Italy; T.J. Martin, Australia; G. Mazzuoli, Italy; L.J. Melton, U.S.; J.D. Ringe, Germany; P. Riis, Denmark; W.A. Peck, U.S.; G. Samsioe, Sweden; and L.E. Shulman, U.S.

Invited experts presenting evidence were: C.H. Chesnut, U.S.; S.R. Cummings, U.S.; P.D. Delmas, France; J.A. Eisman, Australia; H.K. Genant, U.S.; R.P. Heaney, U.S.; C.C. Johnston, U.S.; J.A. Kanis, U.K.; R. Lindsay, U.S.; P.J. Meunier, France; A.M. Parfitt, U.S.; J.-Y. Reginster, Belgium; B.L. Riggs, U.S.; B.J. Riis, Denmark; R.D. Wasnich, U.S.

COMMENTARY

The Role of Soy Products in Reducing Risk of Cancer[1]

Mark Messina, Stephen Barnes*

Since the initial recognition that diet plays a role in the etiology of certain cancers, particularly cancers of the breast and colon, considerable progress has been made in identifying dietary patterns associated with cancer risk. There is general agreement that a high-fat, low-fiber diet, like that consumed by much of the industrialized world, increases cancer risk and that plant-based diets, rich in whole grains, legumes, and fruits and vegetables, are protective. It has been, however, considerably more difficult to identify specific foods, types of food, or components of foods that influence cancer risk.

The recent workshop on The Role of Soy Products in Cancer Prevention, sponsored by the National Cancer Institute, had two objectives: 1) to evaluate the role of soybeans, food products derived from soybeans, and specific components of soybeans in the dietary prevention of cancer and 2) to recommend research initiatives and approaches for further studies of the effect of soy intake on human cancer risk. The meeting was chaired by Stephen Barnes and organized by Mark Messina.

Isoflavones in Cancer Prevention

Kenneth Setchell, Donna Baird, and Barnes discussed the potential role of isoflavones in the prevention of cancer. Setchell reviewed the history of phytoestrogens (*1*), noting that plants were first observed to induce estrus in animals in 1926. Over 300 plants are now known to possess estrogenic activity (*2,3*). In 1946, the infertility observed in Australian sheep that grazed on a certain type of subterranean clover was attributed to the high isoflavone content of this plant (*4*). Ruminal bacteria in these animals convert plant isoflavones into the mammalian isoflavone equol, which, following absorption, may suppress the pituitary gonadotropic axis. Equol, a weak estrogen possessing about 0.2% of the biological activity of estradiol, was first identified in human urine in 1982 by Setchell et al (*5,6*). Setchell's further interest in the potent estrogenic effects of soybean isoflavones was stimulated coincidentally. He discovered that the soy component of diets fed to captive cheetahs, which was added for economic reasons, was responsible for the severe breeding problems in these animals (*6,7*).

Setchell noted that isoflavone metabolism has been studied in humans, although only superficially. In one study, subjects fed 40 g of soy daily were found to have urinary levels of equol as much as 1000-fold higher than baseline values (*8,9*). The low levels of urinary equol in two of the six subjects in this study indicate that the intestinal microflora (*10*) participate in isoflavone metabolism and that isoflavones undergo enterohepatic circulation (*10*). Improved analytical methods (*11,12*) have led to the realization that equol represents only a small fraction of the total amount of isoflavone in urine and that conjugates of the soybean isoflavones daidzein and genistein are the major forms present. The high levels of isoflavone in urine in subjects fed soy suggest that these compounds are likely to elicit a biological response (*13*).

Setchell concluded his presentation with a reminder (*a*) that all weak estrogens can also have antiestrogenic activity; (*b*) that tamoxifen, which has been used therapeutically for breast cancer, is structurally related to some of the phytoestrogens; and (*c*) that vegetarians, who may have a lower risk of certain cancers, excrete higher levels of phytoestrogens. These findings have led to collaborative studies by Barnes, Setchell, and associates (*14*), who used an animal model designed to test the hypothesis that phytoestrogens have a role in reduction of breast cancer risk.

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[1] Report of a workshop held June 26-27, 1990, at the Guest Quarters Hotel in Bethesda, Md. Workshop members were Donna Baird, National Institute of Environmental Health Sciences, Research Triangle Park, NC; Stephen Barnes, University of Alabama at Birmingham, Birmingham, Ala; David L. Brandon, Western Regional Research Center, United States Department of Agriculture, Berkeley, Calif; James A. Duke, Agricultural Research Service, United States Department of Agriculture, Beltsville, Md; Ernst Graf, The Pillsbury Co, Minneapolis, Minn; Ann R. Kennedy, University of Pennsylvania Medical School, Philadelphia; Renee M. Kosslak, Iowa State University, Ames; Irvin E. Liener, University of Minnesota, St. Paul; Mark Messina, National Cancer Institute, Bethesda, Md; Frank L. Meyskens, University of California, Irvine, Calif; A. Venket Rao, University of Toronto, Ontario, Canada; Kenneth D. R. Setchell, Children's Hospital, Cincinnati, Ohio; Bernie F. Szuhaj, Central Soya, Fort Wayne, Ind.

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Barnes began by observing that Oriental women, who have low incidence rates of breast cancer (*15*), consume larger amounts of soy products than do most American women. However, although fertility and reproduction in animals are adversely affected by ingestion of plant isoflavones, the amount of isoflavones in soy products consumed by Oriental women does not appear to affect their reproductive capacity.

Barnes discussed the recent animal study conducted in collaboration with Setchell and other investigators (*14*). In that study, consumption of soybeans significantly decreased chemically induced rodent mammary cancer. Rats were fed one of four soy products: powdered soybean chips consisting of unpurified soybeans, both raw and autoclaved; soy protein isolate composed of 91% protein; soy molasses, a concentrate of the aqueous alcohol extract of soy flour; and aqueous alcohol-extracted soy protein concentrate. All diets were isocaloric and isonitrogenous and produced similar weight gain among the animal groups throughout the study.

The first three products, all of which are rich in isoflavones, inhibited mammary tumorigenesis induced by 7,12-dimethylbenz[*a*]anthracene or methylnitrosourea, while the aqueous alcohol-extracted soy protein concentrate, which had a low content of isoflavones, did not. Whether the soybeans were raw or cooked made no difference in the degree of inhibition of mammary cancer; cooked soybeans were shown to be devoid of protease inhibitor activity.

Barnes said the reduction in levels of mammary tumor estrogen receptors induced by the powdered soybean chips paralleled the inhibition of tumorigenesis and supported the hypothesis that the isoflavones exerted an antiestrogenic effect. Interestingly, however, this was not the case for the soy protein isolate. The decrease in levels of mammary tumor estrogen receptors was smaller than predicted from the degree of tumor inhibition, he said, suggesting that the antiestrogenic effect of isoflavones may not be the primary mechanism responsible for inhibition of tumorigenesis. Therefore, Barnes concluded, the anticarcinogenic activity of isoflavones may not be limited to tumors containing a functional steroid receptor system. Alternative mechanisms of action may include inhibition of the activity of tyrosine protein kinases (eg, epidermal growth factor receptor tyrosine kinase) (*16*), DNA topoisomerase II (*17*), and ribosomal S6 kinase (*18*), as well as induction of specific cytochrome P450s (*19*).

Baird, before describing her recent study of the effects of feeding soy to postmenopausal women (manuscript in preparation), cited the concern of the National Institute of Environmental Health Sciences about the possible effects of low-level environmental estrogens on health. In her study, changes in estrogenic activity in postmenopausal women consuming soy over a 4-week period were examined. Soy was chosen for this study because of its high estrogenic activity (*20,21*), its increasing use in the United States, and the variety of products derived from soy and because soy consumption would not adversely affect nutritional status (*22*). Subjects consumed daily one main soy dish (½ cup of soybeans or 38 g of texturized vegetable protein) and two soy snacks—either soy chips (a roasted soybean product) or a spread for crackers made from the whole soybean. The estimated isoflavone content was about 200 mg/day, the equivalent of about 0.3 mg/day of conjugated steroidal estrogen, assuming that the estrogenic activity of phytoestrogens is about 0.1% that of conjugated estrogen.

Baird said preliminary findings indicate that, compared with control subjects, significantly more women fed soy exhibited an estrogenic response, as demonstrated by an increase in the number of superficial cells of the vaginal epithelium. She remarked that postmenopausal women were chosen for this study because of the decision to examine the estrogenic rather than the antiestrogenic effects of plant phytoestrogens. In premenopausal women with relatively high estrogen levels, the antiestrogenic effects of soybeans may have been observed.

Protease Inhibitors

Ann Kennedy, David Brandon, and Irvin Liener focused their attention on the soybean protease inhibitors. Kennedy reviewed her work, as well as that of others, in the field of protease inhibitors and cancer prevention. She noted that the soybean-derived Bowman-Birk protease inhibitor (BBI) either inhibits or prevents development of experimentally induced colon (*23*), oral (*24*), lung (*25*), liver (*23*), and esophageal cancers (von Hofe E, Newberne P, Kennedy A: unpublished observations). Protease inhibitors, at the levels used in these studies, do not adversely affect animal growth. Kennedy noted that the anticarcinogenic effect of the BBI is thought to stem from its ability to inhibit chymotrypsin activity (*26*), but results also suggest an important role for trypsin inhibition in suppression of the promotional stage of carcinogenesis (*27*). She said in vitro work indicates that protease inhibitors prevent conversion of normal cells to the malignant state even at very late stages in carcinogenesis but that they have no effect on cancerous cells (*28*). Protease inhibitors are unique in that they cause an irreversible suppressive effect on the carcinogenic process. They have also been shown to suppress oncogene expression and to inhibit carcinogen-induced protease activity (*29*).

Kennedy said recent data suggest that the antigrowth effects of raw soybeans commonly attributed to protease inhibitors may actually be due to an unidentified factor(s) (*30*). Furthermore, in human populations consuming soybeans, the connection between pancreatic enlargement and protease inhibitors observed in animals has not been seen. In fact, incidence of pancreatic cancer is decreased in these groups (*31*). Kennedy noted that in vitro comparisons of the pure BBI with an extract of soybeans containing BBI indicate that the activity of the soybean extract could be directly attributable to BBI (*26*). However, she said an in vivo study suggests that the extract may contain an additional anticarcinogenic agent working in conjunction with the BBI (*26*). The extract contains approximately 50% protease inhibitor; the remaining content is unknown, but it may include isoflavones as well as other potential anticarcinogens. Kennedy commented that the lowest effective dietary levels of protease inhibitors used in these animal studies (0.1%) could be achieved by humans by modifying the diet to include soy products.

Brandon discussed the measurement of protease inhibitors in soybeans and soy products, noting the concern of the Agricultural Research Service of the United States Department of Agriculture (USDA) over the possible adverse effects of

protease inhibitor intake in humans, particularly in infants (*32*). Enzyme-linked immunosorbent assays (ELISA), using monoclonal antibodies, have been developed for the measurement of two different protease inhibitors found in soybeans—BBI and Kunitz trypsin inhibitor (KTI) (*33,34*). These procedures are suitable for quantifying residual protease inhibitor levels in foods. A variety of processed soy products, a series of soybean flours derived from seeds in the USDA Soybean Germplasm Collection, and the soybean isoline L81-4590 (lacking KTI) (*35*) have been analyzed. Brandon noted that an important observation from the ELISA analysis of heat-treated soy flours derived from the isoline was that KTI, not BBI, is responsible for the heat-stable activity of commercial soy flour that inhibits trypsin activity (*36,37*). The microenvironment of the soy flour appears to promote heat inactivation of BBI to a greater extent than it affects KTI. This finding contrasts with the results of work showing that BBI is relatively heat stable in the pure form (*38*). Moisture, fat content, the presence of agents that influence changes in disulfide bonds, and interactions with other constituents, such as carbohydrates, appear to influence the denaturation of inhibitors (*39*).

Brandon said analysis of infant formula has revealed that active KTI and BBI, when measured on the basis of weight per gram of protein, are reduced to about 0.1% of their levels in raw soy (*40*). An infant on a diet consisting exclusively of soy formula would consume about 10 mg of active KTI plus BBI per day. In toasted (autoclaved) soy flour, 20%-30% of the KTI activity remains, while all of the BBI is inactivated. Analysis of tofu (soybean curd) has revealed that the protease inhibitor content varied significantly among the samples, from 4 to 30 μg of BBI and from 5 to 16 μg of KTI per gram of product. The protease inhibitor content of several soy protein isolates also varied, as much as 20-fold. Not unexpectedly, there was also a wide variation in the protease inhibitor content among varieties of soybeans. Brandon suggested that food-processing strategies could be combined with genetic approaches to optimize the protease inhibitor content of soy products.

Liener reviewed research on the potential adverse effects of consuming protease inhibitors, first noting that most work has been done with small experimental animals (*41*). Consumption of raw soybeans has two major effects: growth inhibition and pancreatic enlargement. Rats consuming raw soy flour for extended periods develop adenomatous nodules involving acinar cells of the pancreas (*42*). Additionally, raw soy flour consumption potentiates the effect of pancreatic carcinogens (*43*). In a study by Liener et al (*44*), heat treatment of raw soybeans almost completely eliminated this potentiation, while the addition of protease inhibitors to the heated product restored most of the pancreatic enlargement observed with raw soy, suggesting that protease inhibitors are at least partly responsible for pancreatic enlargement.

Liener noted that the varied response to raw soy flour among species is particularly important. Rats, mice, chickens, hamsters, and young, growing guinea pigs all exhibit pancreatic enlargement in response to protease inhibitors, while dogs, pigs, calves, and monkeys do not (*45*). Growth inhibition induced by soybean products is thought to result from a deficiency of the sulfur-containing amino acids caused by the dramatic increases in fecal levels of endogenous protease enzymes, particularly trypsin and chymotrypsin, two enzymes that are rich in these amino acids (*46*).

Commenting that pancreatic enlargement apparently stems from elevated serum levels of the hormone cholecystokinin, Liener commented that pancreatic enzyme secretion is inversely related to the level of trypsin in the intestine, a process regulated by cholecystokinin. This hormone stimulates the pancreas to produce trypsinogen, but because the protease inhibitors combine with trypsin, the suppressive effect of trypsin on intestinal release of cholecystokinin is eliminated (*47*).

Liener raised the question: Can the effects of protease inhibitors in small animals be extrapolated to humans? A negative feedback system in humans has been observed (*48*). Directly supplying BBI or raw soy flour to the duodenum causes an increase in secretion of pancreatic enzymes (*48*) and in blood levels of cholecystokinin (*49*). (BBI, in contrast to KTI, survives in gastric juice.) Despite these observations, he said, it is not possible at this time to accurately assess the health consequences of consuming processed soy products.

Phytosterols and Saponins

A. Venket Rao presented evidence for reduction of colon cancer risk by phytosterols and saponins. Both substances are common constituents of plants, but the concentration in soybeans is particularly high. Phytosterols are structurally similar to the animal sterol cholesterol. They inhibit cholesterol absorption and are almost quantitatively recoverable in fecal material, indicating that very little intestinal absorption occurs (*50*). Soybeans are a major contributor of phytosterols to the diet, particularly β-sitosterol (90 mg/100 g edible portion of the soybean) (*51*). Soybean oil is potentially an important source of phytosterols, but upon refinement and hydrogenation, phytosterol levels are reduced from 315 mg to 217 mg and 132 mg, respectively, per 100 g of oil (*51*). Dietary phytosterol intake among populations differs dramatically; the typical western diet contains about 80 mg/day, while Japanese and vegetarian diets provide about 400 and 345 mg/day, respectively (*52,53*).

In addition to the phytosterols, whole soybeans contain significant amounts of saponins, about 5% of dry weight (*54*), while tofu contains approximately half that much. Saponins are amphophilic compounds having surfactant properties and, like phytosterols, bind to cholesterol and bile acids.

Rao said that while nutritional interest in both phytosterols and saponins has focused on their cholesterol-lowering properties, some data suggest that these compounds may be anticarcinogens. In rats, β-sitosterol-supplemented diets (0.2% by weight) inhibit chemically induced colon cancer (*55*), and phytosterols reduce, in a dose-dependent fashion, cholic acid-induced colon cell proliferation and mitotic activity (*56*). Diets containing phytosterols at 1% by weight are well tolerated by experimental animals (*57*). Dietary saponins from soybeans and other sources have been shown to enhance immunity (*58,59*), are cytotoxic to Sarcoma 37 cells (*60*), inhibit DNA synthesis in tumor cells (*61*), decrease the growth of human epidermoid carcinoma cells (*62*) and human cervical carcinoma cells (*63*), and inhibit Epstein-Barr virus genome expression (*64*). Saponin-sup-

plemented diets (1% by weight), as is the case for the phytosterols, normalize abnormal colonic cell proliferative activity induced by carcinogens (Rao AV: unpublished observations).

Inositol Hexaphosphate

Ernst Graf discussed the rationale for the hypothesis in which inositol-1,2,3,4,5,6-hexaphosphate (IP_6), not fiber, is postulated to be responsible for the inverse correlation between the incidence of colon cancer and the consumption of fiber-rich foods (*65*). When the IP_6 content of cereals, fruits, and vegetables is considered, the international data suggest that there is a greater negative correlation between IP_6 and colon cancer incidence than between fiber and colon cancer incidence. IP_6 is found in a variety of plant foods, particularly cereals, but soybeans are an especially rich source, containing about 1.4% on a dry-weight basis (*66*).

Graf noted that most nutritional interest thus far has focused on the inhibitory effect of IP_6 on mineral absorption. IP_6 forms tight chelates with a variety of polyvalent metals such as calcium, zinc, and iron (*66*). However, he said, the ability to bind metal ions, particularly iron, may provide the basis for the anticarcinogenic effects of this compound. Graf commented that iron may be a key factor, via the Haber-Weiss reaction, in the production of hydroxyl radicals, which are postulated to play a role in the etiology of some cancers (*67*). IP_6 has been shown to limit the oxidant reactivity of transition metals (*66*), to inhibit lipid peroxidation (*67*), and to inhibit experimentally induced colon cancer (*68-73*). It has also been suggested that IP_6, through absorption following dephosphorylation to IP_3, could be an important second messenger involved in the regulation of cell differentiation (*73*).

Phytochemical Variation

James Duke discussed phytochemical variation in soybeans. Duke started by noting that there are over 10 000 named or numbered varieties of the common soybean *Glycine max L.* In these varieties, as one might expect, lies tremendous chemical variation. The genus *Glycine* was originally applied to a distant relative, now known as *Apios americana*, which is an edible root with more protein than is found in potato (*74*).

The isoflavone content of soybeans varies tremendously according to the plant part, variety, year harvested, and geographic location (*75*). Soybean hulls contain only relatively minor amounts of isoflavones, the majority of which occur in the hypocotyl, although one common isoflavone, genistein, is found primarily in the cotyledon (*75*). Equally significant are the reported differences in isoflavone content according to the varieties of soybeans and the year harvested. One study (*75*) reported a threefold variation in total isoflavone content among four varieties of soybeans, while a 30% variation was noted in a single variety of soybeans over a 4-year period. The content of individual isoflavones varied as much as 50%. Not surprisingly, location influences isoflavone content, even within fairly close geographical areas.

Duke noted that chemical variation is not limited to the isoflavones. In some instances as much as a fivefold variation was found among different phenolic acids in soybeans, many of which have also been investigated as potential anticarcinogens.

Isoflavones in Plant Physiology

Renee Kosslak described the role of isoflavones in defense strategies utilized by plants. Plants produce a wide range of products or secondary metabolites thought to enhance their survival (*76*). The isoflavones daidzein and genistein are the major inducers of the nodulation genes in *Bradyrhizobium* bacteria, which form nodules on soybeans (*77*).

The genetic regulation of isoflavone synthesis in plants is not well understood, in part because of the limited number of appropriate mutants affecting this pathway (*78,79*). In soybeans, near-isogenic lines that differ in their root fluorescence are being examined to determine whether they are active in genetic regulation of isoflavone synthesis (*80*). (These differences in root fluorescence in soybeans were first observed in 1934.) There are five loci that affect root fluorescence (*80*), and although specific substances responsible for this property have not been identified, isoflavones are thought to be involved. Preliminary data indicate that the levels of daidzein, genistein, and coumestrol, which is also a phytoestrogen, were either reduced or absent in root extracts from three of the nonfluorescent isolines tested (Kosslak R: unpublished observations).

Kosslak suggested that if future research implicates isoflavones and/or phytoestrogens as important dietary factors in cancer prevention and if the demand for soybean specialty products materializes, it may be possible to manipulate levels of these compounds in soybeans, using root fluorescence as a marker.

Soybean Processing

Bernie Szuhaj briefly discussed soybean processing procedures (*81-83*). Solvent extraction is the primary method of producing soybean products today. Soybeans entering the plant are first cleaned, cracked, and dehulled. Then moisture is added so they can be "flaked," leaving a product that is 3% hypocotyl, 89% cotyledon, and 8% hulls. The oil is removed from the flakes by hexane, producing defatted flakes and soybean oil. From the defatted flakes come a variety of products with a protein content, on a dry-weight basis, that ranges from about 50% for soy flour and grits to about 60%-70% for protein concentrates and about 90% for protein isolates. The primary difference between soy protein concentrates and isolates is the larger percentage of carbohydrate in the soy protein concentrates. Many commercial doughnuts contain soy flour, and, in Europe and Asia, there is particular interest in the use of full-fat soy flours for baking.

Szuhaj noted that most soybean production today goes into animal feed, while the soy protein concentrates and isolates are marketed primarily for their multifunctional properties, such as emulsifying, gelling, fat-binding, texturizing, and dough forming. Soy products play a major role in the food chain. They are added to a wide variety of foods, from cereals to chili. Some

meat products, such as ground beef, contain up to 25% soy. These products have been used in the Armed Forces' canteens since 1983 and in the federal school lunch program.

Discussion

This workshop had two objectives: *1*) to evaluate the relationship between the risk of certain cancers and consumption of soybeans, products derived from soybeans, and/or specific components of soybeans and *2*) to recommend research initiatives aimed at clarifying this relationship. The consensus of the meeting was that there are sufficient data to justify studying the impact of soybean intake on cancer risk in humans.

There were three workshop recommendations. First, future dietary studies involving soybeans should be carried out using soy products rather than isolated compounds, since soybeans appear to contain several potential anticarcinogens. Additionally, because components of food interact, both negatively and positively with each other, the potential benefit of soy products cannot be accurately predicted solely on the basis of the effects of individual soybean components. This does not, however, prohibit future use of isolated soybean components as chemopreventive agents in clinical trials. Second, standardized and improved analytical methods are needed so that the contents of all soy-based materials employed in soybean research, whether soybean fractions or soy products, can be accurately described. This methodology will allow for valid comparisons among studies. Third, basic research on the absorption, metabolism, and physiology of potential anticarcinogens in humans should be conducted. This research will likely help to determine the clinical relevancy of these compounds and to provide a basis for selecting specific soy products for use in future dietary studies.

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Naturally occurring oestrogens in foods—A review

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This review is concerned with the presence of naturally occurring oestrogens in food plants and processed foods. Particular emphasis is placed on isoflavones and coumestans, both of which are true plant oestrogens, and the resorcylic acid lactones, more correctly classified as fungal oestrogens. The metabolism and mode of action of these compounds is discussed and their biological potencies, determined in both *in vivo* and *in vitro* studies, described. Current methods of analysis are indicated and the levels of these oestrogens in food plants, processed foods and feedingstuffs are presented. Botanical, environmental or technological factors affecting the possible intake of plant and fungal oestrogens are mentioned and the hazard associated with such intake is compared with that originating from other dietary or medicinal hormonally active substances. Indications are given of the wide range of common food plants which have been reported to possess oestrogenic (uterotropic) activity, although it is emphasized that in general further work is necessary to substantiate these claims and to confirm the identities of the biologically active principles which have in some cases been proposed. In the concluding section suggestions are made for additional research considered important or necessary in this interesting area.

Introduction

The presence in plants of oestrogens, compounds which induce oestrus in immature animals or interfere with normal reproductive processes, has been known for over half a century. However, the use of plants and plant extracts to control fertility in animals and humans has been recognized since earliest times. In the Orient, for example, the pomegranate has traditional associations with fertility which stretch back over 2000 years. Although many of the plant oestrogens have now been separated, purified and characterized, only occasionally have they been found to be identical with those of animal origin, oestrone (I) and 17β-oestradiol (II) (Hewitt *et al.* 1980) (see figure 1).

In 1954, Bradbury and White listed 53 plants which possessed the capacity to initiate oestrus in animals, but progress in this area was such that only two decades later Farnsworth *et al.* (1975) were able to describe over 300 such plants. In many cases the exact nature of the active principles has not been established but, of the identified compounds, isoflavones and coumestans are the most common. In all, these authors listed 29 plant oestrogens, many of which possessed structural similarity to synthetic diethylstilboestrol (III) (figure 1). Less than half the compounds listed have been reported in plants which are regularly consumed by animals or man. Such plants are listed in table 1 and it may be noted that certain of these, for example legumes and fodder crops, may be consumed in relatively large amounts. Indeed, problems of infertility in livestock (especially sheep) resulting from the grazing of oestrogen-rich pasture or fodders are a serious economic problem in many parts of the world (Hanson *et al.* 1965, Bickoff 1968, Shutt 1976) and have provided the stimulus for much of the

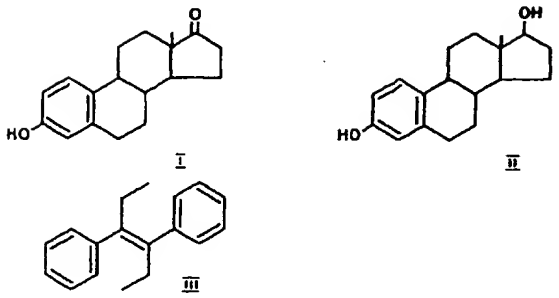


Figure 1. Structures of animal and synthetic oestrogens.

Table 1. Oestrogenic principles of edible plants.

Plant	Common name	Part	Active principle
Avena sativum	oats	seed, meal, sprouts	zearalenone[a] zearalenol[a]
Cicer arietinum	chick pea Bengal gram	seed, seedling	isoflavones isoflavones
Daucus carota var. *sativa*	carrot		3-methyl-6-methoxy-8 -hydroxy,3,4-dihydro isocoumarin[b]
Foeniculum vulgare	fennel	oil	anethole[b]
Glycyrrhiza glabrata	liquorice	root	oestriol, β-sitosterol[b]
Hordeum vulgare	barley	embryo	zearalenone[a]
Humulus lupulus	hops		colupulon[b] lupulon[b] adlupulon[b]
Malus sylvestris	apple	fruit	oestrone
Medicago hispida	toothed medic		isoflavones
Medicago litteralis	barrel medic		coumestrol
Medicago sativa	alfalfa		4-methoxycoumestrol
Oryza sativa	rice	seed, embryo	zearalenone[a] oestrone, oestradiol
Phaseolus vulgaris	French bean	seedling	oestradiol
Phoenix dactylifera	date palm	seed	oestrone
Pimpinella anisum	anise	oil	anethole[b]
Poa pratensis	bluegrass		isoflavones
Prunus avium	cherry	fruit	prunetin
Punica granatum	pomegranate	seed	oestrone
Secale cereale	rye		zearalenone[a]
Sesamum indicum	sesame	meal	zearalenone[a]
Soja max	soya	seed sprouts	isoflavones coumestrol
Sorghum vulgare	sorghum		zearalenone[a]
Triticum vulgare	wheat	flour, seed, germ oil	zearalenone[a]
Trifolium spp.	clovers	leaves stems	coumestrol isoflavones
Vigna sinensis	cowpea		coumestrol
Zea mays	corn		zearalenone[a] zearalenol[a]
	hay		zearalenone[a]

[a] It should be emphasized that zearalenone and zearalenol are not produced by the plant *per se* but may occur on the plant as a result of synthesis by *Fusarta*.

[b] Tentative.

work which has been conducted on plant oestrogens. However, with the exception of those topics of relevance to the presence and effects of plant oestrogens in the human diet, e.g. studies on the analysis and metabolism of oestrogens and of their possible carry-over into the human body via the ingestion of animal products, the role of such compounds in fodders and other animal feedingstuffs will not be considered here.

Additional interest in naturally occurring oestrogens has resulted from the disquiet of scientists, consumers and legislators over the presence in meat and meat products of compounds, such as diethylstilboestrol, designed to improve animal growth and performance (Umberger 1975, McMartin *et al.* 1978). Although the biological activities of such compounds, expressed on a unit weight basis, are very much greater than those of plant oestrogens (see below), under normally regulated conditions their intake into the human body will be very much less. Since any health risk due to dietary factors is a consequence of both biological potency and exposure, there has in recent years been considerable study of plant oestrogens, their metabolism, modes of action and potencies. Such studies have revealed the considerable extent to which genetic, botanical and environmental factors determine the contents of these compounds and also how the processing of the raw plant prior to its consumption can exert similar effects. These studies have, in no small part, benefitted from the development of improved methods of chemical analysis, possessing greatly improved sensitivity and specificity. This paper reviews the more recent advances in these areas and identifies others awaiting additional investigation.

The major plant oestrogens

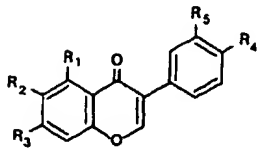
In this review, for convenience, the term 'plant oestrogen' will be used to describe all of the compounds considered in this section, although the resorcylic acid lactones have been referred to elsewhere as *fungal* oestrogens.

The biological effects of plant oestrogens (in the form of the purified compounds or as fresh plants, extracts or processed material) are generally demonstrated by measuring the uterine enlargement of immature female mice or the degree of cornification of the vaginal epithelium. Whereas the former is the more sensitive it lacks the specificity of the latter (Stob 1983); both assays are, however, subject to criticism and misleading results are possible (Emmens 1969).

Examination of table 1 reveals that compounds responsible for the oestrogenic activity mainly fall into three groups, according to their chemical structure. These are (*a*) isoflavones, which in many cases are present in the bound, glycosidic form; (*b*) coumestans; and (*c*) resorcylic acid lactones. A distinction can readily be made between the first two groups and the latter; isoflavones and coumestans are intrinsic plant components, although their levels are dependent upon many factors, including those associated with growth and genetic background. In addition, their levels may also be increased as a direct response to microbial or insect damage. In contrast, the resorcylic acid lactones are products not of the plant *per se*, but of *Fusarium* moulds which are common in the field and flourish in the warm, moist conditions of badly stored grains and other produce. Although other individual compounds possessing oestrogenic activity do occur in food plants, and are considered in the penultimate section of this paper, the major part is concerned with the above groups which are considered separately below.

Isoflavones and isoflavone glucosides

The naturally occurring isoflavones which have been shown to possess oestrogenic activity are (figure 2): daidzein (IV) and genistein (V), their glucosides, daidzin (VI) and



R_1	R_2	R_3	R_4	R_5	
H	H	OH	OH	H	IV
OH	H	OH	OH	H	V
H	H	O-glu	OH	H	VI
OH	H	O-glu	OH	H	VII
H	H	OH	OCH_3	H	VIII
OH	H	OH	OCH_3	H	IX
OH	H	OH	OCH_3	OH	X
OH	H	OCH_3	OH	H	XI
H	H	O-6′acetylglu	OH	H	XII
OH	H	O-6′acetylglu	OH	H	XIII
H	OCH_3	OH	OH	H	XIV
H	OCH_3	O-glu	OH	H	XV

Figure 2. Structures of naturally occurring isoflavones.

genistin (VII), and their 4′-methyl ethers, formononetin (VIII) and biochanin A (IX), respectively; two other active isoflavones, pratensein (X) and prunetin (XI) are of rather limited occurrence. It is possible that other derivatives may also possess uterotropic activity; for example, Japanese workers have isolated the 6′-O-acetyl derivatives of both daidzin and genistin (XII and XIII, respectively) from soyabeans, but they do not appear to have been assayed for their oestrogenic effects (Ohta *et al.* 1979, 1980). It is, however, likely that they are metabolized *in vivo* by ruminants and other animals to daidzin and genistin or their aglycones. Most of the above isoflavones occur in the intact plant in the bound form, as glucosides, but are readily degraded to the aglycone chemically or enzymically during processing, isolation and analysis. Bound isoflavones in clover and related pastures are readily hydrolysed by endogenous glycosidases when the intact plant is crushed (Beck 1964) and such hydrolysis can also occur in animals, and presumably man, in the absence of the plant enzyme. A large number of isoflavones have been isolated from plant species, but only a small number have been shown to possess oestrogenic activity. Moreover, not all isoflavones isolated from plants known to affect oestrus are active; for example, whilst soyabeans possess daidzein, genistein and their glycosides, they may also contain the uterotropically inactive glycitein (XIV) and glycitein-7β-glucoside (XV) (figure 2) (Naim *et al.* 1973).

The biological potencies of the individual isoflavones vary, but all are much less active than animal or synthetic oestrogens. Thus, although genistein is the most potent isoflavone in terms of its effect on mouse uterus (figure 3), it exhibits only 10^{-5} of the activity of diethylstilboestrol. The relative activities of the individual isoflavones vary with both the species and strain of animal used and with the route of administration. In sheep, biochanin A and genistein were about 20 times less active when introduced

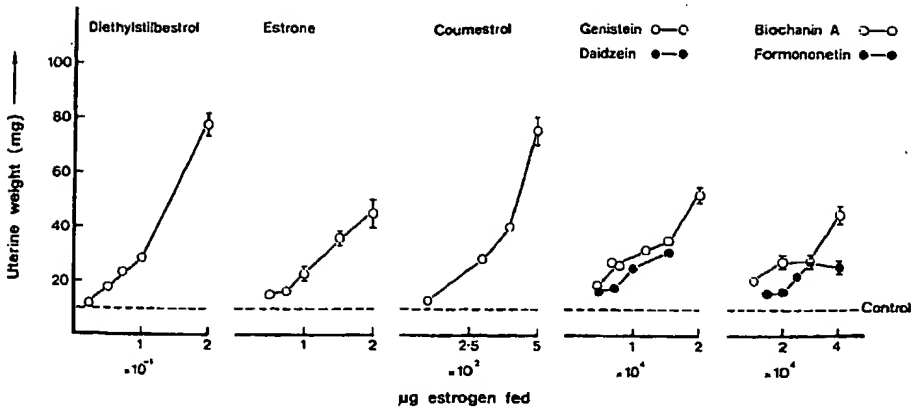


Figure 3. Relative uterotropic potency of diethylstilboestrol, oestrone, coumestrol and isoflavone oestrogens (after Stob 1983).

intraruminally as compared to intramuscular injection, whereas the latter route showed formononetin to be inactive (Braden *et al.* 1976). Genistein was found to be the most active of the isoflavone aglucones tested by oral administration in the mouse (Bickoff *et al.* 1962) and together with its glucoside was equally active when administered subcutaneously (Cheng *et al.* 1955). Differences in responses to other oestrogens between strains of mouse have, however, been reported (Fredericks *et al.* 1981) and recently Farmakalidis and Murphy (1984b) have shown the CD-1 mouse strain to be relatively insensitive to daidzin, genistin and genistein. Comparisons between data arrived at using different strains of mouse are, as the authors point out, thus to be treated with caution. Moreover, there would seem an obvious need to specify, and indeed standardize, the strain of mouse used in the uterotropic assay. Bickoff *et al.* (1962) have demonstrated that dietary isoflavones (daidzein, genistein) possessing a free 4′-hydroxyl group were more uterotropically active in the mouse than their 4′-methyl ethers (formononetin and biochanin A, respectively). The greater potency of genistein compared with daidzein has been attributed to interaction between the OH group and the adjacent carbonyl group of the latter (Bradbury and White 1954). The effect of pratensein is not included in figure 3, but it has been considered to be lower even than formononetin (Wong 1963).

Isoflavones, like the other main groups of plant oestrogens, exhibit an affinity for oestrogen receptor sites (Shutt and Cox 1972, Shutt 1976) and may therefore be considered to function as anti-oestrogens (Martin *et al.* 1978, Verdeal *et al.* 1980). (Anti-oestrogens are thought to exert their effect by decreasing the concentration of cytoplasmic oestrogen receptor and by complexing with the receptor, thus preventing biosynthetic processes associated with tissue development.) The affinities for the binding of genistein to rat, rabbit and sheep uterine cytosol are 1·3, 0·6 and 0·9 respectively (relative to 17β-oestradiol = 100). Other isoflavones are even less active: daidzein exhibits a relative binding affinity of 0·1 and 0·09 for sheep and rat uterine cytosol respectively; biochanin A has an affinity of 0·07 for rat uterine cytosol; and formononetin 0·01 for binding to sheep uterine cytosol (Verdeal and Ryan 1979). The isoflavone metabolites equol, O-desmethylangolensin and angolensin (see below) had relative affinities for sheep uterine cytosol of 0·4, 0·05 and 0·03 respectively (Shutt and Cox 1972).

Based upon the competitive binding to oestrogen receptors in steroid-binding globulins from human breast cancer cells (line MCF-T) the affinities of genistein and formononetin, relative to 17β-oestradiol, are 2 and 0·01 respectively (Martin *et al.* 1978). It seems most likely, as Verdeal and Ryan (1979) have suggested, that transport and metabolic effects are responsible for the apparent discrepancy between the results of the above affinity bioassays and those based upon uterotropic activity. The effects of pure isoflavones in the mouse, rat and sheep are summarized in table 2.

Table 2. Effects of pure isoflavones.[a]

Animal	Compound	Dose	Effect
Mouse	biochanin A	10–40 mg/g diet	uterine hypertrophy
	daidzein	5–15 mg/g diet	uterine hypertrophy
	formononetin	15–40 mg/g diet	uterine hypertrophy
	genistein	5–20 mg/g diet	uterine hypertrophy
	genistein	15 mg/day, diet	infertility, both sexes
	genistein	10 mg injected	displacement of oestradiol from uterine receptors
	genistin	5 mg/day, diet	uterine hypertrophy
	genistin	0·2% diet	infertility, females
	genistin	9–72 mg/day, diet	testes atrophy, depressed growth
Rat	genistein	0·5% diet	testes atrophy, depressed growth
	genistein	0·4 mg, injected	increased protein, phospholipid synthesis in uterus
	genistin	0·5% of diet	testes atrophy, depressed growth
Sheep	biochanin A	1 g, injected	uterine hypertrophy
	formononetin	24 g, injected	uterine hypertrophy
	genestein	1 g, injected	uterine hypertrophy

[a] Full references will be found in Stob (1983), from which this table is taken with permission.

Investigation of the metabolism of isoflavone oestrogens was stimulated by the problem of clover disease in sheep (Bennetts *et al.* 1946). Originally it was assumed that this condition, characterized by a marked loss of fertility, was due to the high levels of isoflavones present in subterranean, and other, clovers. Millington *et al.* (1964) were unable, however, to establish a relationship between the hormonal activity in sheep fed clover and the levels of genistein or biochanin A; a positive relationship was, however, found between the weaker oestrogen, formononetin, and such activity *in vivo*. It is now realized that the reason for this apparently anomalous situation lies in differences in the metabolism of these isoflavones in the digestive tract. Whereas biochanin A and genistein are converted into inactive products, formononetin is metabolized to the isoflavan equol (XVII), and it is this compound in the animal which produces the effect on oestrus (Shutt and Braden 1968). Equol does not, however, appear to be metabolized in the tissues of the sheep (Braden *et al.* 1967). The uterotropic effect of equol is only 10^{-3} that of 17β-oestradiol (Tang and Adams 1980), a potency which is consistent with its relative molar binding affinity to uterine cytosol receptor *in vitro* (Shutt and Cox 1972).

The major pathways which have been elucidated for the metabolism of formononetin (VIII) are shown in figure 4. The primary route, A, involves initial

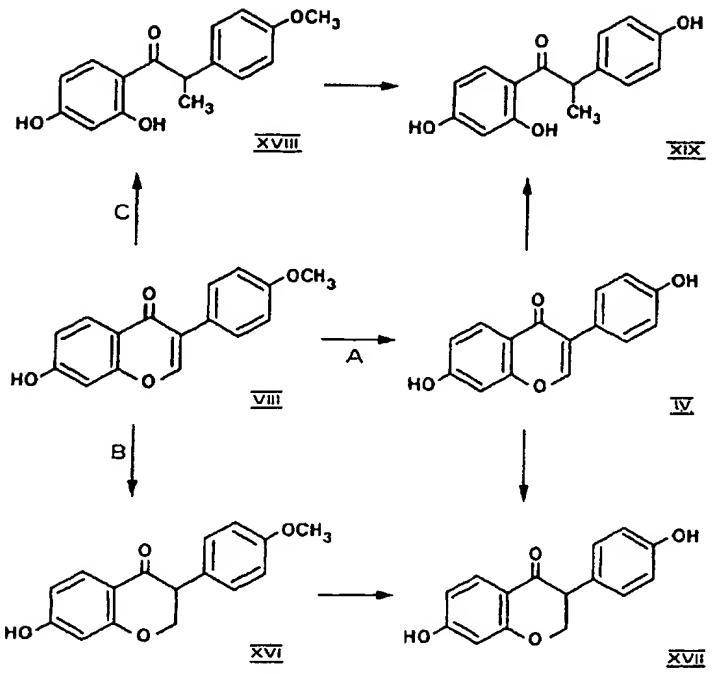


Figure 4. Metabolism of formononetin in the sheep.

demethylation (forming daidzein, IV) and then reduction. A secondary process (B) involves reduction to the 4′-methyl ether of equol (XVI) followed by demethylation. Equol possesses about one half of the affinity for binding to receptor sites of sheep uterine cytosol exhibited by genistein and approximately one quarter of the uterotropic activity of this compound when assayed by intravaginal tetrazolium reduction after oral administration to mice (Shutt and Braden 1968). About 70% of the formononetin ingested by sheep is converted to equol (Shutt *et al.* 1970) and, to a smaller degree, daidzein; in addition, other active metabolites, angolensin (XVIII) and O-desmethylangolensin (XIX) (figure 4, route C) may also be formed (Batterham *et al.* 1971). These compounds are uterotropic in mice and bind to sheep uterine cytosol receptor sites (relative affinities, angolensin 0·03 and O-desmethylangolensin 0·05) (Shutt and Cox 1972). In agreement with the findings of Bickoff *et al.* (1962), referred to above, the oestrogenic activity of the 4′-methyl ether, angolensin, was lower than that of its 4′-desmethyl analogue (Micheli *et al.* 1962).

In marked contrast to the above, biochanin A is metabolized in the sheep via demethylation (to genistein, V) and thence, by ring cleavage (presumably involving the intermediate phenyl-α-methylbenzyl ketone) to the oestrogenically inactive *p*-ethylphenol (XX, figure 5) (Braden *et al.* 1967).

Comparative studies in sheep and cattle revealed the latter to metabolize formononetin more rapidly and also to be more effective in conjugating isoflavones and their metabolites (Braden *et al.* 1971). In sheep the metabolism of biochanin A and genistein in the rumen is initially low but increases significantly over the first few days of grazing on clover and related forages; this is paralleled by a reduction in the hormonal effect of these crops. In marked contrast, the rate of formononetin degradation is not affected by time to any great extent, hence the pasture retains its oestrogenicity

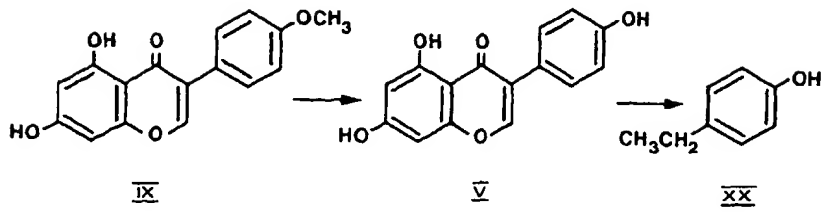


Figure 5. Metabolism of biochanin A in the sheep.

(Lindsay and Francis 1969). Provided that the livestock are removed from such pasture the oestrogenic effects are reversible. However, continued grazing will lead to permanent physiological changes of the reproductive tract (Lindner 1976). Equol has been identified in the urine of goats, rats and hens and in each case was considered to result from dietary isoflavone precursors, rather than being present in the diet *per se*.

Shutt *et al.* (1970) have observed the metabolism of isoflavones in sheep to proceed rapidly; for example, 1 g of biochanin A plus genistein was metabolized in about 90 min. Moreover, the data presented suggested that the initial demethylation (A in figure 4), rather than the reduction, was the rate-limiting step. Equol does not appear to suffer extensive degradation in the rumen, but is readily absorbed therefrom (residence time 1·7 h). There is a suggestion that residence times for isoflavones may be reduced under grazing conditions, a consequence of which may be the less complete metabolism of isoflavones in the rumen, a greater concentration of genistein resulting and/or a decreased production of equol from formononetin (Shutt *et al.* 1970). Consequently the oestrogenic activity, and effect, of such pasture in livestock depends upon the fine balance of isoflavone metabolism *in vivo*.

In contrast to the metabolites of steroidal oestrogens, isoflavones are readily conjugated as glucuronides and excreted. According to Shutt *et al.* (1967) circulating isoflavones are almost exclusively present in the form of biologically inactive glucuronides, although small amounts of the free compounds and their sulphoconjugates, which can yield the free compounds *in vivo*, may also occur. A similar situation has been observed in man (Axelson *et al.* 1982). The plasma content of dietary isoflavones in sheep following the feeding of red or subterranean clovers was maximal 30 min after feeding and thereafter rapidly declined; the content of equol increased from 4 to 10 μg/100 ml plasma between 30 and 150 min after feeding, whilst the measured conjugated equol in plasma was very much higher (300–400 μg/100 ml) and was largely independent of feeding time (Shutt *et al.* 1967).

Equol was first reported in human urine by Axelson *et al.* (1982). Total daily excretion levels of two male subjects were 10·9 and 35·2 μg, whilst those of four female subjects ranged from 10·7 to 43·3 μg. In most cases $\geqslant$99·8% of the measured equol was excreted as the glucuronide, but in two subjects 5·7 and 9·9% was bound as the sulphoconjugate. Independently Adlercreutz *et al.* (1982) reported that there was no significant difference in the daily urinary excretion of equol by post-menopausal women who were vegetarians (mean 35·8 μg, range 0–113 μg), omnivores (mean 35·8 μg, range 0–102 μg) or suffering from breast cancer (mean 27·2 μg, range 0–74 μg). The maximum mean daily excretion measured was 565 μg over a three-day period, and at such a level the authors considered that a biological effect might result. Subsequently Bannwart *et al.* (1984) identified both daidzein and equol monoglucuronides in the urine of five female subjects. The levels found in four vegetarian subjects (two pre- and two post-menopausal) were much greater than that measured in the single pre-menopausal,

omnivorous subject (daidzein: average 396·6 μg/l, range 96·0–1108 μg/l compared with 21·6 μg/l; equol: average 4207 μg/l, range 1493–9663 μg/l compared with 46·0 μg/l). The variation in the vegetarian subjects was ascribed to differences in the composition of the diet. Apples, cherries, potatoes, garlic, hops and soya products were mentioned as the most probable sources of dietary oestrogenic compounds. A less important source of these compounds was considered to be products obtained from animals which had been fed oestrogen-containing forage. This seems probable, although only limited data is available upon which to base a judgement. According to Lindner (1967) the levels of such isoflavones accumulating in the adipose tissue of sheep (1 μg/g) was too low to present a serious health hazard. The effects of cooking and/or processing would, moreover, seem likely to reduce this figure further.

Recent work has emphasized the importance of soya as a source of dietary isoflavones (Axelson *et al.* 1984). Two healthy subjects were given 40 g of commercial texturized soya in place of meat, daily for 5 days. Urinary excretion of equol was found to increase 100–1000 fold (figure 6) and traces of daidzein glucuronides were also observed. Quantitatively similar results were observed in rats, approximately 100 μg of equol being excreted per gram of soya flour ingested. The figure for soya oil is much less (5 μg/g), indicating that little, if any, isoflavones are removed from soya during processing of the oil (see below). This result is of interest also since Vague *et al.* (1957) have reported cornification of the vaginal epithelium to occur in post-menstrual women following the administration of 100 g corn or olive oil per day for 10 days. The uterotropic effect of soya meal and soya-based rations in laboratory animals and poultry is well documented (Drane *et al.* 1980).

Setchell *et al.* (1984) have recently shown that certain people excreted little or no equol in the urine when fed 40 g of commercial soya protein daily for 5 days. The reasons for this behaviour are unclear, although it appears to be unrelated to the sex of the subject; the authors suggest that the rate of formation of equol was dependent upon dietary-related factors, such as the composition of the intestinal microflora, the intestinal transit time and variability in the redox level of the large intestine. These

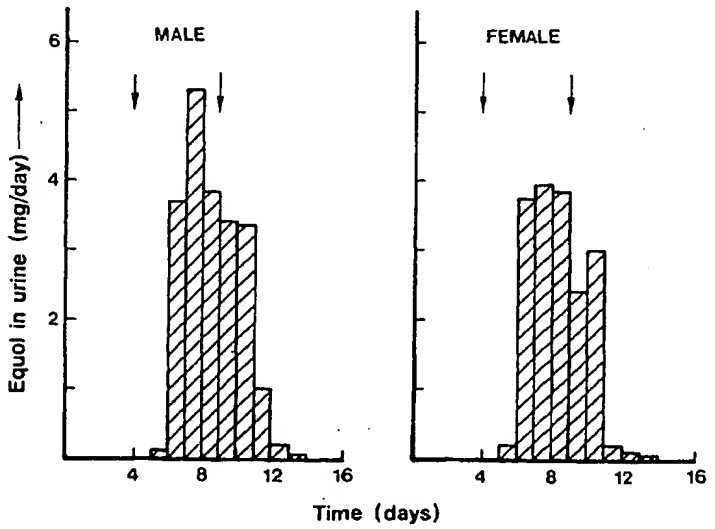


Figure 6. Daily urinary excretion of equol in humans (from Axelson *et al.* 1984). The arrows mark the period over which soya protein, 40 g/day, was fed.

workers also demonstrated that human faecal flora were able to degrade soya-rich broth components (presumably daidzin and daidzein) to equol. As the authors point out, it would be of dubious value to extrapolate the above findings, based upon six subjects, two of whom were obvious non-responders, to the population at large. These results do, however, emphasize the need for further study into the factors affecting phytoestrogen metabolism in man, the metabolic fate of dietary oestrogens in non-responders, and the variation in the rate of phytoestrogen metabolism in larger populations. The latter may in turn lead to the identification of particular 'at-risk' groups within the population at large.

Axelson and Setchell (1981) were unable to determine equol in the urine of germ-free rats fed a commercial, soya-containing ration. Glucosidases capable of converting isoflavone glycosides to the uterotropically active aglucones have been identified in man, as have enzymes which have been shown to carry out the conversion of daidzin to equol (Axelson *et al.* 1984). Although no evidence has yet been presented it would be expected that genistin→genistein→*p*-ethylphenol would represent a similar (but *de*toxifying) metabolic pathway in man.

The improvement of methods for the detection and quantification of isoflavones and their metabolites in plant material and biological samples has been of great importance in the development of an understanding of their chemical and biological properties. Such analysis has been effected by a variety of techniques, including paper chromatography (Markham 1975), thin layer chromatography (Beck 1964), gas chromatography (Naim *et al.* 1974), high-performance liquid chromatography, spectrophotometry, fluorimetry and immunoassay. Gas chromatographic methods, either alone or linked to mass spectrometry, have involved the prior derivatization of the molecules by converting free —OH groups to trimethylsilyl ethers or trifluoroacetyl esters (Naim *et al.* 1973). Gas chromatography-mass spectrometry procedures such as single ion monitoring have allowed very low levels of isoflavone metabolites to be measured (Bannwart *et al.* 1984, Axelson *et al.* 1984), and using conventional gas chromatography procedures the former authors have demonstrated a detection limit of 1 μg equol/24 h urine sample. With such low levels, the isolation, extraction and concentration of the compound(s) of interest from the bulk sample is of paramount importance. The use of reversed-phase silica for preliminary clean-up has proved especially useful for the extraction of isoflavones and equol (and their conjugates) from urine, following which enzyme hydrolysis and ion exchange clean-up processes are employed (Axelson *et al.* 1984, Bannwart *et al.* 1984). Use of DEAE Sephadex (base form) enables a degree of separation between mono- and diphenolic species to be effected (Axelson *et al.* 1982).

The advantage of high-performance liquid chromatography techniques is that the samples can be examined without the need for derivatization; under such conditions both free compounds and conjugates may be analysed directly. Following the original report of Kallela and Saastamoinen (1978), a number of techniques have been described, which almost invariably use reversed-phase systems. Methods developed for the analysis of isoflavones in clover and other fodder crops usually rely upon the facile hydrolysis of the glucosides during plant maceration and extraction such that the isoflavone aglucones are separated and quantified. Petterson and Kiessling (1984) and Sachse (1984) both include chemical hydrolysis prior to sample analysis. Free isoflavones and glucosides are readily determined in soya by high-performance liquid chromatography and, of the methods described, the present authors favour that of Eldridge (1982a) in which all of the likely soya isoflavones are separated, an internal

standard is included and no problems of co-eluting impurities are encountered. The latter severely limits the usefulness of a semi-preparative method for the isolation of daidzin and genistin, reducing the loading capacity to an extent that conventional chromatography (using Sephadex LH20) was of comparable efficiency (Farmakalidis and Murphy 1984a).

The analysis of soyabean (meal) and fractions have almost invariably revealed the presence of daidzin, daidzein, genistin, genistein, glycitin-7β-glucoside and glycitein, the latter two being uterotropically inactive and for this reason not included in table 3. Small amounts of formononetin were also claimed to be present by Shemesh *et al.* (unpublished, cited in Lindner 1976), but details of the method were not given; in the absence of any independent confirmation and bearing in mind the obvious differences between the results of these workers and others (table 3) for the levels of the other isoflavones, this report should be treated with caution. It is generally held that the major proportion of soyabean isoflavones are present as glucosides (table 3), but as has been indicated these are readily degraded by intestinal bacteria prior to metabolism, conjugation and excretion. Bickoff *et al.* (1962) have reported that 8 mg of genistein (or 10 mg of daidzein) was the minimum dose needed to induce a hormonal response in mice; hence the oestrogenic effect of soyabean meal and soyabean-containing commercial rations on poultry and laboratory animals is readily understood, especially when it is further realized that biologically significant levels of coumestrol and its methyl ethers may also be present.

Much research on the isoflavones of pasture and forage crops has demonstrated that many factors (e.g. the physiological age of the plant, its genetic origin, climatic and environmental factors associated with growth) can affect the ultimate content of these compounds in the plant (Bickoff 1968, Rossiter and Beck 1967), and more recent work has shown these factors also to be important in soya. However, additional consideration must be taken of the effect of subsequent processing, especially as it relates to human food ingredients.

Eldridge and Kwolek (1983) have shown that the defatting of full-fat soya does not remove isoflavones or their glucosides, contrary to the earlier claim of Booth *et al.* (1960). Support for this later finding comes from the work of Axelson *et al.* (1984) referred to above. Analysis of soyabean hull (8% by weight), hypocotyl (2%) and cotyledon (90%) fractions revealed isoflavone contents of 10–20 mg/100 g, 1405–1750 mg/100 g and 319–808 mg/100 g respectively. It should be noted that coumestrol is concentrated primarily in the hull and testa portions (Lookhart 1979). Daidzin and glycitin account for more than 95% of the total isoflavone content of the hypocotyl, whereas in the cotyledon the latter is almost absent and genistin predominates. Eldridge (1982b) found that soya protein concentrate (containing 70% protein) prepared by aqueous leaching contained higher levels of isoflavones (247 and 317 mg/100 g) than were present when an aqueous alcohol process was used (16 and 43 mg/100 g). Soya protein isolates, containing 90% protein, although obtained by a variety of unspecified procedures, contained similar isoflavone contents (103–145 mg/100 g), most of which was genistin and genistein. Combined levels of daidzin and daidzein, yielding equol on metabolism, ranged between 24 and 51 mg/100 g. Seo and Morr (1984) found a commercial protein isolate to contain 96 mg isoflavones/100 g. Whilst in general agreement with these findings, Murphy *et al.* (1982) observed the level of isoflavone glucosides in soyabeans to decrease substantially on germination, during protein isolation or when calcium-precipitated tofu was prepared. There appeared, however, to be no corresponding increase in the free forms of these isoflavones. According to György *et al.* (1964)

Table 3. Oestrogenic isoflavone content of soya and its products.

Sample	Daidzin (mg/100 g)	Daidzein (mg/100 g)	Genistin (mg/100 g)	Genistein (mg/100 g)	Formononetin (mg/100 g)	Reference
Soyabean meal	62	48	127	40		Eldridge (1982a)
Soyabean meal	11·7, 0	0, 2·2	74·7, 102·4	4·0, 2·4		Murphy (1982)
Soyabean meal	56·7, 56·1	4·9, 14·5	65·5, 81·3	9·7, 18·7		Pettersson and Kiessling (1984)
Soyabean meal	42	17·8	151	108		Pratt and Birac (1979)
Soyabean flakes	59·6 ± 8	5·6 ± 0·7	215 ± 9	6·7 ± 8		Seo and Morr (1984)
Soyabean flour	48–77	8–48	58–154	4–46		Eldridge (1982)
Soyabean cake		30 ± 5		18·6 ± 2·7	4·3 ± 2	Shemesh *et al.* (in Lindner 1976)
Soyabean flakes	114	2·5	188·5	4·4		Eldridge and Kwolek (1983)
Soya-based animal ration	7		42–45	7		Murphy *et al.* (1982)

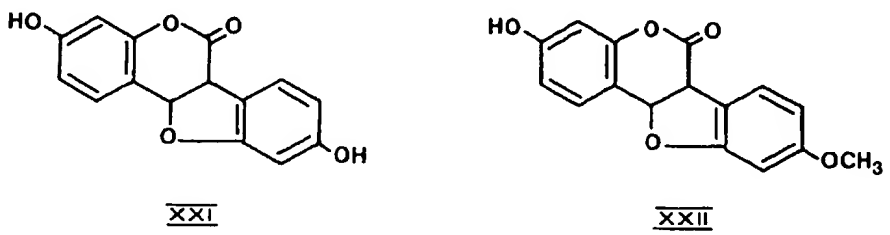
daidzin and genistin are hydrolyzed by *Rhizopus oryzae* during the fermentation of soyabeans to produce tempeh. Defatted soya flakes contained 287 mg isoflavones/100 g (Seo and Morr 1984) and this was decreased by various protein isolation procedures to 203 mg/100 g (acid precipitation), 53 mg/100 g (dialysis), 8·3 mg/100 g (ion exchange) and 6·1 mg/100 g (activated charcoal treatment). A commercial sample of soya protein hydrolysate contained genistein and daidzein contents of 54 and 15·2 mg/100 g, respectively; animal rations containing soya hydrolysates were also observed to possess very low levels of isoflavones (Murphy 1982). Germinated bengal gram (*Cicer arientanum*) was found to contain biochanin A and formononetin at levels of 71 and 77 mg/100 g (Dziedzic and Dick 1982) and 98·6 mg and 44·1 mg/100 g (Sharma 1979a), respectively. The latter worker also identified daidzein (5·1 mg/100 g).

Bartholomew and Ryan (1980) found daidzein, genistein, formononetin and biochanin A all to be non-mutagenic when screened using the *Salmonella*/mammalian microsome assay, the behaviour of the first two compounds being in agreement with the findings of Sugimura *et al.* (1977), and confirmed by Murphy and Glatz (in Murphy 1982).

Isoflavone aglucones have been shown to be responsible in part for the antioxidant activity of soyabeans and their products (György *et al.* 1964, Pratt and Birac 1979, Pratt *et al.* 1981). These compounds also contribute to the astringent and bitter tastes of defatted soyabean (How and Morr 1982) and soy protein products (Huang *et al.* 1981). Soyabean isoflavones possess marked antifungal activity, whereas the glucosides are almost without action (Naim *et al.* 1974). Sharma (1979b) has demonstrated that biochanin A, formononetin and pratensein possess hypolipidaemic activity in the albino rat, but daidzein (and genistein (Ollis 1962)) was inactive. It was considered that this, at least in part, explained the hypocholesterolaemic activity of the black gram and navy bean (Saraswati Devi and Kurup 1972, Hellendoom 1976).

Coumestans

Coumestans possess structures exhibiting close similarity to those of isoflavones to which they are biosynthetically related. A relatively large number of these compounds have been isolated from plants (Wong 1975), but only a few have been shown to possess uterotropic activity. For example, Verdeal and Ryan (1979) list eight coumestans which have been identified in alfalfa, only two of which possess such activity. These compounds, coumestrol (7,12-dihydroxycoumestan, XXI) and 4′-methoxycoumestrol (7-hydroxy-12-methoxycoumestan, XXII) (figure 7) are the most common of this class of oestrogen and have been reported in alfalfa, ladino clover and other fodder crops where their presence is associated with widespread problems of animal performance (Stob 1983). According to Hanson *et al.* (1965), over 90% of the oestrogenic activity of potent dehydrated alfalfa samples was due to its coumestrol content and Lookhart



XXI XXII

Figure 7. Structure of coumestans.

(1980) has found serious oestrogenic effects to result from feeding cattle haylage containing 37 mg coumestrol/kg. Bickoff *et al.* (1960) and others (Micheli *et al.* 1962) have investigated the effect of structural changes in the coumestan molecule on its hormonal activity. Phenolic groups in the 7,12 positions were important; thus the 7-methyl ether and 12-methyl ether (XXII) possessed only 54% and 15%, respectively, of the uterotropic activity of coumestrol itself when administered orally to mice. 7,12-Diacetoxycoumestrol was almost as active as the parent compound when administered in the same manner, presumably reflecting the lability of the acetoxy groupings *in vivo*.

As may be seen from figure 1, the uterotropic potency of coumestrol in the mouse is greater than that of the isoflavones and, as with the latter, variation occurs according to species and means of administration. Braden *et al.* (1967) found coumestrol (administered intraruminally) to be 15 times more active than the most potent isoflavone and it is even more potent when injected intramuscularly. In the mouse, coumestrol is 35 times more active (and its diacetate 24 times more active) than genistein, but still possesses less than 0·03% of the activity of diethylstilboestrol (Bickoff *et al.* 1962). The coumestans, like the isoflavones, bind competitively to mammalian oestrogen receptor sites and are more active when assayed in this manner. The relative binding efficiency (that of 17β-oestradiol = 100) of coumestrol has been reported as 1·4 (rabbit uterine cytosol, Shemesh *et al.* 1972), 4·9 (sheep uterine cytosol, Shutt and Cox 1972), 4·9 (rat uterine cytosol, Verdeal *et al.* 1980) and 19·7 (calf uterine cytosol, Lee *et al.* 1977). When tested in human cancer cell preparations the relative affinity of coumestrol was measured as 9·8 (Martin *et al.* 1978). According to Fredericks *et al.* (1981) coumestrol may exert its effect on fertility *in vivo* by inhibiting follicle stimulating hormone. Little is known about the metabolism of coumestrol; Kelly (1972) has found the compound to be rendered less active in sheep over a period of 7–14 days. Whilst this might be due to the formation of less active metabolites, the chemical nature of which is obscure, more recent work suggests an alternative explanation. Coumestrol is conjugated *in vivo*, but to a rather lower extent than the isoflavones. Thus Kelly and Lindsay (1978) found between 20% and 40% of the total coumestrol in sheep's plasma to be present in the free form (compared to less than 10% in the case of the isoflavones) (Shutt *et al.* 1967). Significantly the concentration of free coumestrol in sheep's plasma remained constant over 16 days, during which time the animals became biologically less sensitive to the oestrogenic effects of this compound. The loss of sensitivity, moreover, appeared to be related to the amount of dietary coumestan and the period of exposure. Further work is needed to clarify the factors underlying these interesting observations. The biological effects of administering coumestrol to animals is shown in table 4.

Coumestrol has been found in a range of plant products commonly consumed by man (table 5). The highest levels were noted in sprouts of alfalfa and, especially, soyabean (Knuckles *et al.* 1976b). Legume sprouts and shoots have in recent years been consumed in increasing amounts by certain sections of the population of the UK and other western countries. It would seem prudent to conduct a more detailed study of the coumestrol (and isoflavone) contents of these materials using modern analytical methods. In the aforementioned work, Knuckles *et al.* used paper chromatography allied to fluorimetric detection and quantification (Knuckles *et al.* 1976a); at the present time, however, the best method of analysis would appear to be high-performance liquid chromatography (Lookhart *et al.* 1978, 1980) using ultraviolet or fluorimetric detection. By judicious choice of mobile and stationary phases it is also possible to monitor isoflavones and coumestrol simultaneously (Pettersson and Kiessling 1984).

Table 4. Effects of pure coumestans and zearalenone.[a]

Animal	Compound	Dose	Effect
Mouse	coumestrol	100–500 μg/g diet	uterine hypertrophy
	coumestrol	500 μg/g diet	antigonadotropic
Rat	coumestrol	1 mg injected, 5 days neonatally	persistent oestrus syndrome
	coumestrol diacetate	125 μg injected	increased protein and phospholipid synthesis in uterus
Sheep	coumestrol	12 mg injected, 1·4 g intraruminally	uterine hypertrophy
Mouse	zearalenone	10 μg/g diet	uterine hypertrophy
		20 μg injected	uterine hypertrophy
Rat	zearalenone	1 mg, oral	uterine hypertrophy
		600 μg topical to skin	uterine hypertrophy
Swine	zearalenone	1–50 mg daily, oral	hypertrophy vulva, vagina, uterus and mammae; metaplasia of cervical epithelial cells
		100 μg/g diet	infertility
		25–100 μg/g diet	infertility, nymphomania, pseudopregnancy, reduced litter size, smaller pigs, malformations, juvenile hyperoestrogenism, probable fetal resorption
Chicken	zearalenone	300–800 μg/g diet	hypertrophy of vent, oviducts and cloacal bursa, eversion of cloaca
Turkey	zearalenone	300–800 μg/g diet	hypertrophy of vent, oviducts and cloacal bursa, eversion of cloaca
Monkey	zearalenone	14 or 56 μg/kg injected	stimulation, LH[b] surge
		14 μg/kg injected	serum LH depression
		400 μg daily, orally for 4 days	serum LH depression

[a] Full references will be found in Stob (1983), from which this table is taken with permission.
[b] LH = luteinizing hormone.

Table 5. Coumestrol content of plant products.[a]

Product	Coumestrol content (μg/100 g dry weight)
Alfalfa sprouts (fresh)	500
Soyabean sprouts (fresh)	7110
Soyabeans (dry)	120
Defatted soyabean meal (dry)	40
Soyabean concentrate	20
Soyabean isolate	60
Frozen green beans	100
Frozen snow beans	60
Frozen green peas	40
Frozen Brussels sprouts	40
Dried red beans	40
Dried split peas	30
Frozen spinach leaf	10

[a] Data from Knuckles *et al.* (1976) with permission.

The coumestrol content of plant material has been observed to vary with a variety of factors (Bickoff *et al.* 1969). For example, Hanson *et al.* (1965) have shown that of alfalfa to be affected, to various degrees, by variety, stage of growth, cutting, the year and location and, to a significant degree, by the presence of disease. Coumestrol has been observed to accumulate in alfalfa and other legumes following insect (Loper 1968) or fungal attack (Loper 1968, Loper and Hanson 1964, Stuthman *et al.* 1966, Loper *et al.* 1967). According to Sherwood *et al.* (1970) coumestrol was not translocated from the infected area to other parts of the plant. Whereas coumestrol in undamaged, non-infected plants was metabolized via the isoflavone pathway (Grisebach and Barz 1963, 1964), the origins of the coumestrol biosynthesized as a result of such insect or fungal damage is unknown.

Concern over the presence of coumestans in alfalfa and ladino clover has resulted from the reduced reproductive performance of animals maintained on such fodder (Hanson *et al.* 1965, Bickoff *et al.* 1969) and both breeding programmes and improved husbandry practices have been initiated to reduce the extent of the problem. Of the latter, treatment with agrochemicals can minimize the pest and fungal attack which results in accumulation of coumestrols and other plant phenolics; moreover, the intake of coumestrol by animals can also be reduced by the feeding of immature plants in which the coumestrol content is known to be lower than in the mature plant. In the absence of any information concerning the amount, if any, of coumestans which enter the human body indirectly via the residues in animal products and milk obtained from livestock grazing on oestrogenic pasture, concern over the intake of these compounds by man is centred mainly upon their presence in common food plants (table 4), vegetable protein and 'health' products.

Leaf protein concentrate has been suggested as a source of protein for humans, and methods have been described for its preparation from alfalfa (Köhler *et al.* 1968, Edwards *et al.* 1975). The effect of such processing on the coumestrol content has been examined by Knuckles *et al.* (1976b). Relatively little of the original coumestan content of the alfalfa (11–118 mg/kg) was removed in the solubles during the early stages of the processing. Protein concentrates possessing 9–14 mg coumestrol/kg were obtained by commercial-type processing in which heat coagulation and washing was carried out under acid conditions (pH 4·5–6·5), whereas if the medium was kept alkaline (pH 8·5–9·5) the coumestrol content was much lower (3 mg/kg) due to the greater solubility of the oestrogen under these conditions. Diafiltered alfalfa leaf protein concentrate possessed a coumestrol content of only 0·4 mg/kg (measured as freeze-dried powder). Since the coumestrol content of diseased or damaged alfalfa leaves may exceed 1000 mg/kg, i.e. 10–100 times that of undamaged tissue, it is clearly important that the quality of the materials selected for processing be maintained as high as possible.

Alfalfa and other leguminous products have been widely marketed in recent years as health foods, tonics and supplements. Recently, Elakovich and Hampton (1984) have analysed commercial alfalfa tablets and found these to contain 20–194 μg coumestrol/g, equivalent on a daily dosage basis to 1–2 mg of coumestrol. The effect of long-term exposure to such levels (together with that of any isoflavone oestrogens which may also be present) cannot yet be ascertained. However, this work clearly points to the desirability of monitoring the contents of physiologically active substances in health products since the 'recommended' doses (if stated) are frequently exceeded and the products may not be covered by the same legislative controls as foods and feeding-stuffs.

Coumestrol has been observed to possess tumour-promoting activity similar to that of 17β-oestradiol and diethylstilboestrol for dimethylbenzanthracene-induced rat mammary tumours (Verdeal *et al.* 1980). However, Bartholomew and Ryan (1980) have reported this compound to be non-mutagenic in the Ames test. Both coumestrol and its 4′-methyl ether had been shown to possess weak antifungal activity (Van Etten 1976).

Resorcylic acid lactones

Unlike the previous two groups of plant oestrogens, the resorcylic acid lactones are not intrinsic components of food plants but are secondary mould metabolites of fungal species, principally *Fusarium*, e.g. *F. roseum* var. *graminearum* (*Gibberella zeae*) which are common field organisms which also proliferate in poorly stored grains, oil seeds and hay (Caldwell *et al.* 1970, Eugenio *et al.* 1970, Sherwood and Peberdy 1972, Abbas *et al.* 1984). There have been a number of detailed reviews on the chemistry (Shipchandler 1975), production and biological activity (Mirocha *et al.* 1971, 1977, Mirocha and Christensen 1974, Pathre and Mirocha 1976, Hidy *et al.* 1977, Betina 1984) of these compounds and a comprehensive coverage of these and other aspects of *Fusarium* moulds is now available (Moss and Smith 1984). The economic losses associated with the feeding, especially to swine and cattle, of rations containing such mould-damaged produce have rightly meant that emphasis is primarily placed on the effects of such compounds on livestock, rather than on humans. However, since there is, at least in principle, the possibility of these compounds being carried over into humans via the consumption of animal products, and as many grain and cereal products are now formulated directly for human consumption, it is appropriate to consider the levels of such compounds likely to enter the human body and, thereby, assess the likely risk from such compounds.

The most common oestrogen of this group is zearalenone (6-(10-hydroxy-6-oxo-*trans*-1-undecenyl) β-resorcylic acid lactone, XXIII). The reduced compound, zearalanol (XXVI, figure 8), has been marketed as a growth promoter in sheep and bovines. The main metabolites of zearalenone are the epimeric β- and α-zearalenols (XXV and XXIV). Other compounds have been identified (Verdeal and Ryan 1979), but in general little is known about their biological activity. Zearalenone is usually described as a mycotoxin (F-2 toxin) but some reviewers consider this as inappropriate (Stob 1983). There have been a number of cases reported where the feeding of *Fusarium*-infected rations have caused death, abortion and other serious physiological disorders in livestock and poultry, and in some cases these were attributed to the presence of zearalenone. It is possible that other, more toxic, substances were also present, since such moulds normally produce a number of mycotoxins simultaneously; these may include trichothecenes, such as T-2 toxin, deoxynivalenol and diacetoxyscirpenol. As Stob (1983) has stated, the involvement of zearalenone in some of the more distressing symptoms associated with the feeding of *Fusarium*-infected rations should be treated with circumspection and the role of zearalenone itself should be demonstrated in controlled feeding trials, where such additional compounds can be excluded. The observed LD_{50} of zearalenone is certainly far removed from those of other mycotoxins, being 5, 10 and 20 g/kg in female guinea pigs, rats and mice respectively. For these and other reasons, Stob (1983) has suggested the terms 'mycoestrogen', 'fungal oestrogen' or 'oestrogenic metabolite' as being more appropriate.

Zearalenone, zearalenol and zearalanol have been found to bind to mammalian

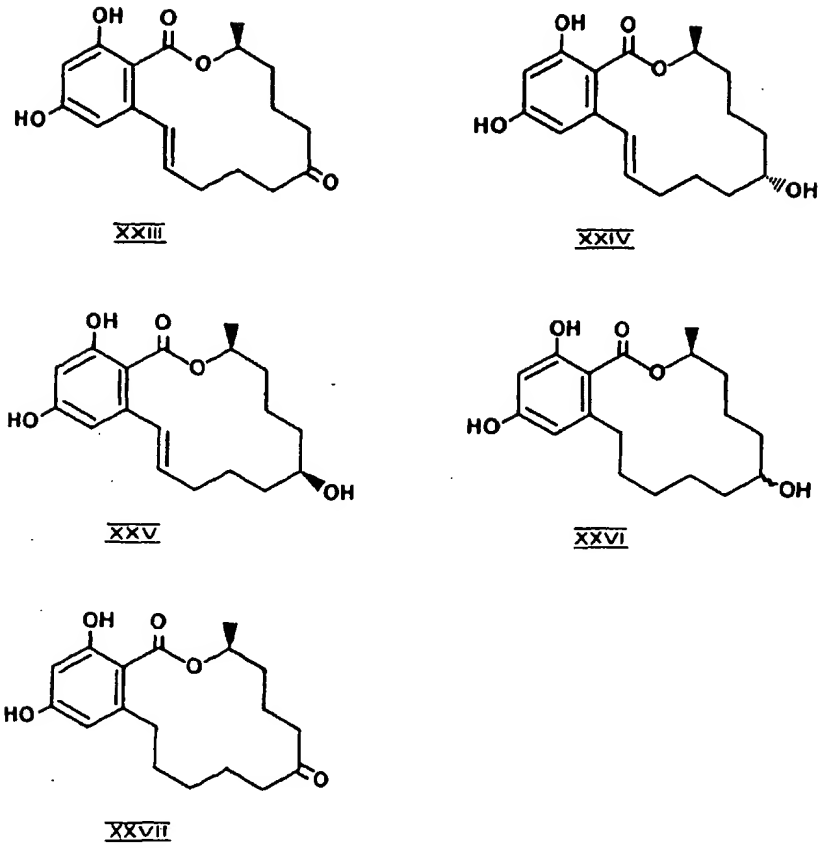


Figure 8.

oestrogen receptor sites. Kiang *et al.* (1978) showed these to bind to uterine cytosol and nuclear receptors in the order *cis*-zearalenone (not naturally occurring) > *trans*-zearalenone > zearalenol (stereochemistry unspecified) > zearalanol. All four compounds almost completely inhibited the binding of 17β-oestradiol at a ratio of 100:1. Katzenellenbogen *et al.* (1979) found α-zearalenol to be more active than either β-zearalenol or zearalanone when measured by competitive or direct binding assays using rat uterine cytosol receptors. The former compound was observed to possess 13·6% and 15% of the effect of 17β-oestradiol upon competitive and direct binding analysis, respectively.

It has been suggested (Ueno and Tashiro 1981) that the oestrogenic effect of zearalenone is due to its metabolism to zearalenol, and this suggestion has been supported by more recent work (Sheehan *et al.* 1984). Despite the structural dissimilarity between zearalenone and 17β-oestradiol, as Duax *et al.* (1984) have pointed out, there is considerable similarity between their respective hydrophobic bulk. Zearalenone binds to rat hepatic cytosol oestrogen receptors (Powell-Jones *et al.* 1981) as well as to those of rat uterus, with which it has been found to bind more strongly than the isoflavones but less strongly than coumestrol (Verdeal *et al.* 1980). Radio-labelled zearalenone, injected intravenously into mice, was found to be bound to oestrogen target organs, e.g. uterus, intestinal testicular cells and ovarian follicles (Appelgren *et al.* 1982). Studies by Martin *et al.* (1978) showed that zearalenone was less potent than

either isoflavones or coumestrol when assayed by competitive binding to human breast cancer cell oestrogen receptors. The uterotropic activity of zearalenone has been demonstrated by various workers (Stob 1983)—for example, when administered by mouth it was 10^3 times less active in the mouse than was diethylstilboestrol (figure 9). By subcutaneous injection in the same species, the compound was 500 times less active than 17β-oestradiol (Katzenellenbogen *et al.* 1979). Mirocha *et al.* (1978) have shown *cis*-zearalenone to possess stronger uterotropic activity than the natural *trans*-isomer; *cis*- and *trans*-zearalenols were found to be of comparable activity by the same workers.

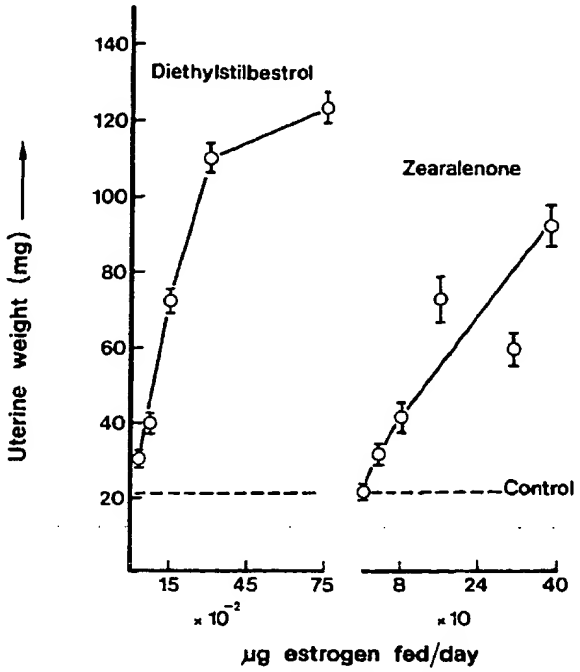


Figure 9. Relative uterotropic activity of orally administered diethylstilboestrol and zearalenone (after Stob 1983).

The biological activity of zearalenone in animals is shown in table 4. Data on the oestrogenic potency of zearalenone in rats has been obtained by Kumagai and Shimizu (1982); by uterotropic assay this compound possessed less than 0·1% of the activity of 17β-oestradiol, a figure in agreement with that resulting from estimation of vaginal cornification following systemic administration. In contrast, the vaginal cornification bioassay indicated that zearalenone possessed about 1% of the activity of 17β-oestradiol when administered locally. Furthermore, neonatal exposure to zearalenone produced anovulatory sterility in the rat, the potency being 10% that of 17β-oestradiol. There is considerable evidence that pigs are especially sensitive to zearalenone (Mirocha *et al.* 1974, Chang *et al.* 1979) with hormonal effects resulting from as little as 1–5 mg/kg diet. According to Chang *et al.* (1979), the inclusion of 25–100 mg zearalenone/kg in the ration of sows led to multiple reproductive deficiencies, including infertility, reduced litter size and weight and hyperoestrogenism. Zearalenone and zearalanol have been shown to increase weight gain when implanted subcutaneously in sheep (Hidy *et al.* 1977) and subsequent study demonstrated the latter to be especially

effective in steers and heifers (Willemart and Bouffault 1983). Further work also resulted in synthetic zearalanol implants being produced commercially.

In primates, orally administered zearalenone was 80 or 160 times less active than 17β-oestradiol or diethylstilboestrol, respectively, in inhibiting synthesis and release of gonadotropins (luteinizing hormones) from the anterior pituitary (Hobson *et al.* 1977), but the effect is much greater if the compounds are administered by subcutaneous injection and all three compounds are then of comparable effectiveness. In humans, 75–100 mg of zearalenone/day have been reported to be effective in the treatment of post-menopausal syndrome in women (Utian 1973, Hidy and Baldwin 1976b) and, according to Hidy *et al.* (1977), the clinically effective daily dose of zearalanol for such purposes is 50–75 mg, a practical result which was in agreement with that inferred from animal experiments. Both zearalanol and zearalenone are effective as oral contraceptive agents in humans (Hidy *et al.* 1976a).

According to unpublished work cited by Hidy *et al.* (1977), the major metabolite of orally administered zearalenone in the sheep was β-zearalenol (XXV) which was shown to possess only 25% of the oestrogenic activity of the parent compound. Ueno and Tashiro (1981) observed only small amounts of this metabolite in rat faeces, the main product being the epimer, α-zearalenol (XXIV), which was three times more uterotropically active than zearalenone and also bound more strongly to mammalian uterine cytosol receptor. α-Zearalenol may be present in both the free and conjugated (β-glucuronate) forms (Kiessling and Pettersson 1978, Olsen *et al.* 1981) and conjugation of zearalenone itself may also represent a significant detoxification process (Kiessling and Pettersson 1978, Olsen *et al.* 1981) in the rat. Rumen microbes have been observed to metabolize zearalenone into α- (mainly) and β-zearalenols and it has been suggested that an additional explanation of the oestrogenic effect of zearalenone-zearalenol metabolism is the interruption of normal steroid metabolism via the necessary involvement of hydroxysteroid dehydrogenase (Kiessling and Pettersson 1978, Olsen *et al.* 1981). As has been mentioned earlier, zearalanol has been used to improve weight gain of livestock. The main metabolite of this compound in a wide range of species, including man, is zearalanone (XXVII); both compounds have been found in the free and bound forms (Hidy *et al.* 1977).

According to Dixon and Russell (1983), when four cattle were implanted with 36 mg of zearalanol, mean maximum urine levels were 13·5 μg/l (22 days after implantation) and declined to 2·9 μg/l by day 69. Two experiments were conducted with sheep, who received 12 mg zeralanol implanted into the base of the ear. Mean maximum urine levels were reached after 35 days (45 μg/l) and 56 days (90 μg/l) in the two experiments, and thereafter declined to 26 μg/l (day 42) and 11·7 μg/l (day 70), respectively. Ruddick *et al.* (1976) reported zearalenone to be teratogenic although more recent work (Davis *et al.* 1977, Wardell *et al.* 1982) could not confirm this.

Numerous methods have been described for the analysis of zearalenone (Gilbert 1984), including thin layer and paper chromatography (with colourimetric or ultraviolet detection) (Caldwell *et al.* 1970), gas chromatography (utilizing the trimethylsilyl- or pentafluoropropionate derivatives) (Steele *et al.* 1976, Holder *et al.* 1977), gas chromatography-mass spectrometry (Mirocha *et al.* 1974, Scott *et al.* 1978) and high-performance liquid chromatography (Scott *et al.* 1978, Cohen and Lapointe 1980). Thin layer and high-performance liquid chromatography have also been used to separate zearalenone and its metabolites in biological samples (Kiessling *et al.* 1984, Ueno and Tashiro 1981). The detection limit for zearalenone using high-performance liquid chromatography, with Spherisorb 5 μm column and fluorescence detection, was

5 μg/kg in corn flakes (although a second high-performance liquid chromatography column was needed to remove an interfering compound in other corn products) and 10 μg/kg in corn (Scott *et al.* 1978, Ware and Thorpe 1978, respectively). For purposes of routine screening, simpler techniques using thin layer chromatography have been developed. With Fast Violet B as spray reagent, detection limits of 20 μg/kg (Scott *et al.* 1978) and 80 μg/kg (Swanson *et al.* 1984) have been reported for zearalenone in corn and corn-based foods. The latter workers also considered the method amenable for the qualitative, but not quantitative, screening of zearalenol (detection limit 200 μg/kg). Immunological techniques have been developed for the detection and quantification of zearalanol (Dixon 1980, Dixon and Russell 1983, Thouvenot and Morfin 1983) but since related compounds, such as zearalanone and zearalenone, may possess significant cross-reactivity towards the antiserum, a preliminary separation with high-performance liquid chromatography has been recommended (Jansen *et al.* 1984).

The extent of the contamination of grain crops with *Fusarium* species may be considerable. In 1972, 38 out of 223 corn samples from areas in the USA where such contamination was suspected or expected were found to contain zearalenone, the levels ranging from 100 to 5000 μg/kg (Eppley *et al.* 1974). A similar study the following year revealed zearalenone levels of 38–294 μg/kg in 19 out of a total of 315 marketable corn samples (Stoloff *et al.* 1976). There was clear evidence of localized regional occurrence with 10% of the samples from the Corn Belt (17 out of 169) being affected. The same workers also measured zearalenone contents of 97–10 400 μg/kg in 57 samples of obviously damaged corn. The results of other surveys of wheat, grain sorghum, soyabeans and corn have been summarized by Bennett and Shotwell (1979). Zearalenone has been detected in six samples of Mexican corn intended for human consumption, but the levels were not quoted (Mirocha *et al.* 1972). Of 293 samples of the 1982 Australian maize crop recently examined (Blaney *et al.* 1984), 85% contained zearalenol; the mean concentration was 170 μg/kg but four samples possessed in excess of 1000 μg/kg. Côté *et al.* (1984) found 40 out of 342 feed samples, obtained in 1981 from the area around Illinois and suspected of causing or contributing to animal health problems, to contain zearalenone. Levels ranged from 100 to 8000 μg/kg, with a mean of 660 μg/kg. In Canada, problems associated with *Fusarium* infection of corn and other crops would seem to occur predominantly in Ontario (Andrews *et al.* 1981). Analysis of suspected samples over the period 1972–1977 revealed some 10% (214 out of 2022) to possess zearalenone, levels ranging from 10 to 141 000 μg/kg, the mean being 3850 μg/kg. Zearalenone, deoxynivalenol and, apparently for the first time, aflatoxin B_1, have recently been identified in commercial wheat samples from the mid-western USA. Of a total of 33 samples examined, zearalenone was present in trace amounts in two samples and, in another three, at levels of 35, 90 and 115 μg/kg (Hagler *et al.* 1984).

There is some disagreement over the effectiveness of chemical treatments for detoxification of zearalenone-contaminated grain. An American patent (Tamas and Wöller 1977) describes either 3–6% aqueous hydrogen peroxide or ammonium hydroxide as effective, but the removal of zearalenone was not quantified. However, unpublished work, referred to by Bennett and Shotwell (1979), found the ammoniation process used for removal of aflatoxins to have no effect on zearalenone levels. More recently, Kallela and Saastamoinen (1981) have shown the farm grain preservative 'Gasol' to have a beneficial effect in reducing the levels of zearalenone in stored grains.

A considerable amount of the world grain crops is used as human food sources, either directly or after processing. In many parts of the world such use represents the

major part of the crops' utilization. There have been a number of reports of zearalenone being found in southern African foods, drinks and raw materials. Thus levels of 100–800 μg/kg were measured in corn used for the brewing of Zambian beer (Lovelace and Nyathi 1977) with an average of 920 μg/kg (maximum 4600 μg/kg) being found in such beers and 800–4000 μg/kg in the corn malt used in the brewing process. Of 55 samples of sour drinks, porridges and beers from Swaziland, six were found to contain zearalenone (referred to in Bennett and Shotwell 1979) at levels between 800 and 5300 μg/kg. Of local beers from Lesotho, 12% of the 140 samples examined also contained this oestrogen (300–2000 μg/kg). Rather lower levels were found in Lesotho beer by Martin and Gilman (1976) (approximately 50 μg/kg) and samples of maize porridge, sorghum malt were also found to be contaminated (Martin 1974). MacDonald and Raemakers (1974) found zearalenone in South African maize samples. Together with zearalenone, the presence of other, more toxic, metabolites may be expected (Bennett and Shotwell 1979) and although the climate in southern Africa might be expected not to be such as to facilitate such mould growth as might occur in other parts of the world, Marasas *et al.* (1977) have found strains of *F. graminearum* in southern Africa capable of producing deoxynivalenol, and possibly other mycotoxins.

According to Stoloff and Dalrymple (1977), zearalenone was not detected in the primary or by-products from dry milling operations. Bennett *et al.* (1976, 1978) have examined the effects of processing on naturally contaminated corn. Wet milling was found to concentrate the oestrogen in the gluten fraction with lesser amounts being found in the milling solubles, fibre and germ respectively. The starch fraction was free of zearalenone. Dry milling led to a two- to three-fold concentration of the zearalenone in the germ. Both milling processes led to a concentration of the zearalenone into fractions used as animal feedingstuffs.

Scott *et al.* (1978) have examined various corn products for zearalenone using both high-performance liquid chromatography and gas chromatography. Largest amounts were found in a sample of cornmeal (26 μg/kg), although two other samples contained no detectable amounts. Frozen corn contained 2 μg/kg, corn chips 0 and 2 μg/kg, popcorn 0 and 7 μg/kg and three samples of cornflakes 0, 0·4 and 14 μg/kg respectively. The carry-over of zearalenone in cattle consuming naturally infected wheat rations has been studied by Shreeve *et al.* (1979). Concentrates (385–1925 μg zearalenone/kg) were fed to two cows for 7 weeks. No zearalenone (detection limit 4 μg/kg) residues were detected in muscle, kidney, liver, serum, milk or urine. The result should be interpreted with some caution bearing in mind the number of animals used and the inability of the analytical method used to detect zearalenone metabolites. The study also revealed apparent indications of interactions between dietary fungal metabolites which would warrant further examination. Mirocha (1981) has detected α- and β-zearalenol (16–76 μg/kg) in the milk of a cow following the oral dosage of [^{3}H]zearalenone. Palyusik *et al.* (1980) have described the results of feeding two lactating sows a diet containing pure zearalenone (40 mg/kg). In addition to various physiological effects attributable to the oestrogenic effect of this compound, analysis of the milk from these animals showed mainly β-zearalenol (> 80% of original toxin) and α-zearalenol (~15%) with only traces (0·5–1·3%) of unchanged zearalenone. The highest concentration of zearalenol found in milk was 0·79 p.p.m. The authors reported that the metabolites could be detected in the milk samples within 2 days of feeding the zearalenone and were still present 5 days after it had been removed from the diet.

Calculations quoted by Lovelace and Nyathi (1977) give possible daily intakes of zearalenone of 450 μg and 170 μg for rural farmers in Southern Province and

inhabitants of Lusaka, respectively. The figure for certain individuals is certainly much higher. Marasas *et al.* (1979), on the basis of animal data, considered that 500 μg/kg was a biologically significant dose of zearalenone. However, as has been indicated, there is a considerable variation in sensitivity between species and the toxicity of zearalenone in man is unknown, but based upon data from other primates (Hobson *et al.* 1977) is probably low. Ueno and Kubota (1976) suggested that zearalenone was mutagenic to a recombination-deficient line of *Bacillus subtilis*, but this could not be confirmed by Wehner *et al.* (1978), using *Salmonella typhimurium*.

Schoental (1979) has suggested that zearalenone and other *Fusarium* mycotoxins may have a role in the aetiology of tumours of the digestive tract and gonads in animals and man, and there has been speculation (see Martin and Keen 1978) that dietary oestrogens might be implicated in the high incidence of cervical cancer in certain areas of Southern Africa, e.g. Swaziland and Lesotho.

Mouldy corn from areas of the Transkei associated with high and low incidences of oesophageal cancer has been examined for mycotoxins (Marasas *et al.* 1979). Pooled samples from these areas showed no significant differences in the extent and nature of the *Fusarium* species present. However, when four sub-samples of hand-selected, visibly *Fusarium*-infected kernels were analysed, significant differences in the nature and extent of the infection were observed. All of the sub-samples contained zearalenone (ranging from 1500 to 10 000 μg/kg) but the mean level of the samples from the high-incidence area was 5750 μg/kg compared to 2750 μg/kg from the low-incidence area. Even larger differences were noted in the levels of deoxynivalenol, being 250 μg/kg and 2500 μg/kg in the low-incidence and high-incidence areas respectively. The authors concluded that before the potential threat to human health of these *Fusarium* metabolites in mouldy corn could be evaluated more detailed information was needed on their chronic effects and on whether any additive or synergistic effects might occur.

Other compounds claimed to possess oestrogenic activity

Examination of table 1 reveals additional compounds which have been claimed, with varying degrees of supportive evidence, to be responsible for the oestrogenic activity of the individual plant species shown. For example, Stob (1983) has suggested that the hormonal activity of carrots (Ferrando *et al.* 1961) may be related to the presence of 3-methyl-6-methoxy-8-hydroxy-3,4-dihydroisocoumarin (XXVIII, figure 10). However, this compound was isolated from cold-stored carrots and was apparently absent in the freshly harvested root (Sondheimer 1957). Little, if anything, is known about the effect of such storage on the uterotropic effect of this vegetable. Anethole (XXIX) was suggested by Zondek and Bergmann (1938) to be responsible for the oestrogenic activity of essential oils of fennel and anise, but more recent physiological studies on this compound (Sangster *et al.* 1984a, b) have not supported this. Three structurally related bitter acids, colupulon (XXX), lupulon (XXXI) and adlupulon (XXXII) have been identified in hops and proposed as the oestrogenic principles therein (Zenisek and Bednar 1960).

Feldman *et al.* (1982) described a protein in bakers' yeast (*Saccharomyces cerevisiae*) capable of binding 17β-oestradiol with high affinity; moreover, a chloroform extract of the same yeast cells was found to bind competitively to mammalian oestrogen receptor cells *in vitro*. Subsequently the same group (Feldman *et al.* 1984) showed this extract to possess uterotropic activity. If these findings are confirmed, high priority should be given to the isolation and identification of the active component(s), given the extensive use of this material in baking and fermentation. Only when its potency has

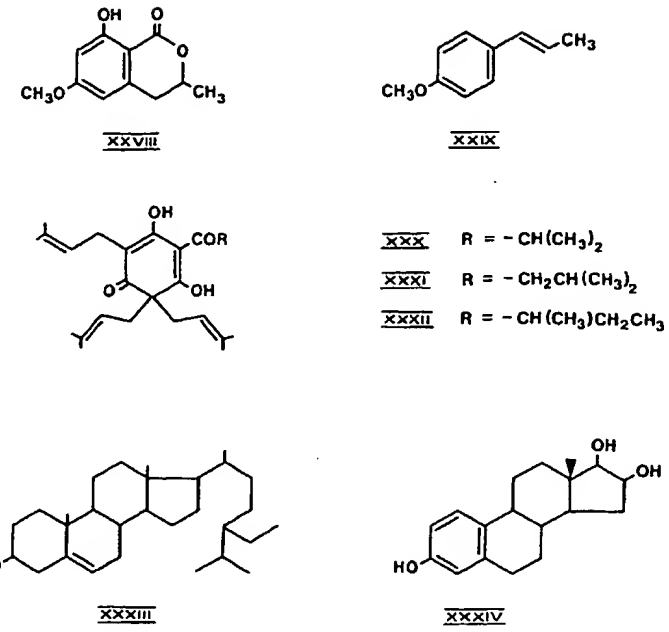


Figure 10.

been determined and its levels in food products have been established can the full significance of the above findings be ascertained.

According to Hassan *et al.* (1964), β-sitosterol (XXXIII) was one of the factors responsible for the hormonal activity of liquorice, although this has been questioned. Certainly if this and related compounds were to be confirmed as possessing such activity, then their ubiquity might well explain the hormonal properties of a range of common food plants, including onion and garlic and certain vegetable oils (Booth *et al.* 1960). Rather earlier, Costello and Lynn (1950) had tentatively identified the steroidal oestrogen, oestriol (XXXIV), as being present in liquorice. The role, and indeed the presence, of such steroidal hormones in the plant kingdom has been the subject of considerable controversy (Hewitt *et al.* 1980). As these workers have emphasized, early investigations into the hormonally active principles of plants were limited by relatively crude means of fractionation, isolation, characterization and bioassay. Consequently many of the initial claims for the occurrence of steroidal oestrogens in the plant kingdom were treated with scepticism. In 1966, Bennett *et al.* identified oestrone in date palm pollen by thin layer chromatography, and it was subsequently isolated from the same source (Amin *et al.* 1969). Pomegranate seed was claimed to be another source of this compound, with levels of 17 mg/kg being reported (Heftmann *et al.* 1966).

Whilst the presence of oestrone was confirmed by Dean *et al.* (1971), the measured levels were very much lower (4 μg/kg). 17β-Oestradiol could not be detected. In other cases (see Hewitt *et al.* 1980), workers were unable to isolate steroidal oestrogens from plant sources despite previous claims to the contrary. Such irreproducibility, limitations in analytical technique and methodology and the overriding concern that presence of such compounds, even when proved conclusively, might be a result of contamination meant that fundamental questions about the natural occurrence of such compounds in plants remained until recently (Van Rompuy and Zeevaar 1979), and the

failure of these workers to identify steroidal oestrogens in plant extracts using sophisticated modern techniques clearly identifies this area as a rewarding one for further interdisciplinary study. Work described in detail by Hewitt *et al.* (1980), using radioisotope incorporation studies and sensitive gas chromatography-mass spectrometric techniques, unequivocally revealed the presence of oestrone and oestradiol in French bean seedlings.

Residues of pesticides and insecticides may also be a source of uterotropically active compounds in the human diet. It has long been known that DDT and its analogues exhibit such activity (Fisher *et al.* 1952, Welch and Conney 1968) and recently Loeber and van Velsen (1984) have shown β-HCH, an isomer of lindane and a component of technical HCH, to have uterotropic activity. Although very weak (2×10^{-5} that of 17α-ethynyloestradiol), little is known about the effects of long-term exposure to trace amounts of such compounds.

Overview

Amongst the plants consumed by humans which have been reported to possess oestrogenic activity are onion, garlic, coffee, apple, parsley, sage, rhubarb, potato, radish, pea, cucumber, sugar beet, cabbage and mustard. These reports, originating from the early work of Dohrn *et al.* (1926) and Löve and Löve (1945), did not identify any of the active components, and were based upon methods of analysis which are now recognized to have limitations. Nevertheless it would seem desirable to re-examine some of these food plants using modern methods of analysis, in particular those like potato and cabbage, which are consumed regularly in relatively large amounts. The widespread use of vegetable oils also suggests that the claims that these are uterotropic be re-examined. Because of the evident variation in sensitivity to oestrogens exhibited by different species and strains of animal it would be desirable to standardize the uterotropic assay so that results from different laboratories and on different commodities/food plants could be more readily compared.

Inspection of table 6, taken from Verdeal and Ryan (1979), would seem to suggest that there is little risk associated with the intake of plant oestrogens. This is not necessarily the case, however, since little is known about the effects of long-term low-level exposure to these compounds (or their metabolites). Studies with human subjects would be desirable to determine whether or not normal levels of intake are associated with detectable physiological changes. This might provide objective predictions of the nature and extent of any changes which might occur in particular

Table 6. Human exposure to exogenous oestrogens.[a]

Source	Estimate of possible daily dose (μg diethylstilboestrol equivalents)
Morning-after pill	50 000
Birth control pill	2500
Post-hysterectomy replacement therapy	500–1000
Post-menopausal therapy	500
100 g beef liver (0·5 p.p.b. diethylstilboestrol)	0·05
100 g wheat (2 p.p.m. zearalenone)	0·2
20 g (d.w.) soyabean sprouts (70 p.p.m. coumestrol)	0·5
100 g French beans (2–10 p.p.b. oestradiol)	0·03–0·15

[a] Data from Verdeal and Ryan (1979) with permission.

'at-risk' sections of the population. Moreover, consideration should be taken of any medium or long-term changes in dietary habits which might be expected to increase the intake of such phytoestrogens; the increasing use of vegetable proteins in general and soya protein in particular and the introduction of soya milk products for infant feeding are two such examples (Setchell *et al.* 1984).

The importance of metabolic studies in determining the likely oestrogenic effect associated with the ingestion of isoflavones and resorcylic acid lactones is obvious, the effect depending as it does on the extent of that metabolism and the individual potencies of the metabolic products. The metabolism of the coumestans should be examined and the activities of the major isolated metabolites determined. Further studies should also be conducted on equol; for example, examining its effect *in vivo* and *in vitro*. Such studies will obviously depend upon the availability of methods of analysis for both the parent compounds and the metabolites. The examination of the possible carry-over of resorcylic acid lactones, and metabolites, following the feeding of *Fusarium*-infected diets or the implantation of zearalanol as growth stimulant would be desirable using such methods. The possible synergistic effect of different plant oestrogens or of plant and synthetic oestrogens should not be discounted (Kotsonis *et al.* 1975).

Attention should be given to programmes designed to limit or reduce the intake of plant oestrogens, whether by judicious selection of plant varieties or the optimization and improvement of agronomic, storage and processing conditions. Notwithstanding the inherent difficulties, a detailed study of the dietary factors associated with the high incidence of certain cancers in Southern Africa might provide useful information as to the role of dietary oestrogens. Indeed, a fuller understanding of the biological basis of the hormonal activity of the plant oestrogens considered above, particularly in farm animals and primates, would seem long overdue.

Future progress in this interesting and challenging area will largely depend upon the integrated efforts of workers from a variety of disciplines, including chemists, biochemists, toxicologists, pathologists, food technologists and plant breeders. As such it would seem to be particularly suitable for support from national and international food and health agencies.

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Use of a Mammalian Cell Culture Benzo(*a*)pyrene Metabolism Assay for the Detection of Potential Anticarcinogens from Natural Products: Inhibition of Metabolism by Biochanin A, an Isoflavone from *Trifolium pratense* L.[1]

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ABSTRACT

Based on the epidemiological evidence for a relationship between consumption of certain foods and decreased cancer incidence in humans, an assay was developed to screen and fractionate plant extracts for chemopreventive potential. This assay measures effects on the metabolism of [^{3}H]benzo(*a*)pyrene [B(a)P] in hamster embryo cell cultures. Screening of several plant extracts has generated a number of activity leads. The 95% ethyl alcohol extract of one of these actives, *Trifolium pratense* L. Leguminosae, red clover, significantly inhibited the metabolism of B(a)P and decreased the level of binding of B(a)P to DNA by 30 to 40%. Using activity-directed fractionation by solvent partitioning and then silica gel chromatography, a major active compound was isolated and identified as the isoflavone, biochanin A. The pure compound decreased the metabolism of B(a)P by 54% in comparison to control cultures and decreased B(a)P-DNA binding by 37 to 50% at a dose of 25 μg/ml. These studies demonstrate that the hydrocarbon metabolism assay can detect and guide the fractionation of potential anticarcinogens from plants. The ability of the isoflavone biochanin A to inhibit carcinogen activation in cells in culture suggests that *in vivo* studies of this compound as a potential chemopreventive agent are warranted.

INTRODUCTION

Humans are exposed to numerous carcinogens and mutagens daily, some avoidable (such as cigarette smoking) and some virtually unavoidable (diet, environmental pollution, oxygen radicals). The diet has been shown to have a profound effect on the incidence and location of various human cancers worldwide (1, 2), and epidemiological studies suggest that certain dietary components may help to prevent cancer induction. This prophylaxis has been termed cancer chemoprevention. Wattenberg (3) has demonstrated that such agents may inhibit cancer induction by a number of mechanisms. One of the more common mechanisms is through inducing alterations in the enzymatic activation or detoxification of carcinogens.

Although many biological assays have been used to examine the chemopreventive potential of various chemicals, there have been relatively few studies using activity-directed fractionation to isolate active compounds from plants. In addition, it is impractical to use *in vivo* models to guide these procedures. Wattenberg *et al.* (4) used an activity-directed fractionation procedure based upon induction of aryl hydrocarbon hydroxylase activity in the liver and intestinal mucosa of Sprague-Dawley rats to isolate and identify several indoles from cruciferous vegetables. Kaweol and caffestol palmitates were isolated from green coffee beans (5) based upon an assay that measured the increase in glutathione S-transferase activity in liver and intestinal mucosa of mice. Practical assays for activity-directed fractionation of active plants must be rapid, sensitive, convenient, and capable of detecting alterations in carcinogen metabolism. In this paper, we describe the development and application of an assay that measures effects on the metabolism of [^{3}H]benzo(*a*)pyrene, a widespread environmental carcinogen, in early passage cultures of Syrian hamster embryo cells (6). The chemical and analytical procedures developed for activity-directed fractionation of antineoplastic compounds from plants (7, 8) were adapted to the isolation and identification of potential anticarcinogens from food and food plants, such as red clover extracts, which significantly inhibited the metabolism of benzo(*a*)pyrene and binding of B(a)P[3] metabolites to DNA.

MATERIALS AND METHODS

Spectroscopy and Chromatography. ^{1}H NMR in deuteriochloroform was performed using a Varian XL-200, and ^{13}C NMR in deuteriochloroform was measured on a Chemagnetics A-200 spectrometer. EI and CI mass spectra were obtained on a Finnigan 4023 quadrupole mass spectrometer. High-resolution mass spectra were recorded on a Kratos MS 50. The IR spectrum was performed on a Beckman IR-33 using a KBr pellet. UV spectra were measured on a Beckman DU-7 in methyl alcohol using sodium methoxide, $AlCl_3$, HCl, and sodium acetate as UV shift reagents.

For flash column chromatography EM 9385 Silica Gel 60 was used for the adsorbent. Radial chromatography was performed on a Chromatotron Model 7924 using a 1-, 2-, or 4-mm rotor with EM 7749 Silica Gel 60 PF 254 as adsorbent. TLC plates were Merck 5714 Silica Gel 60 F_{254}.

Cell Culture Toxicity Assay. Hamster embryo cell cultures were prepared and grown as described previously (6). Tertiary cultures were plated in 60-mm plastic dishes (Falcon) (5×10^5 cells), and 24 h later the test compound was added at 10-fold dilutions from 500 μg/ml of medium to 0.05 μg/ml for 24 h. At that time the cultures which were approximately 70% confluent were examined microscopically and subjectively evaluated for the percentage of the cells dividing and the cell density. The highest noninhibitory dose was selected for metabolism studies.

B(a)P Metabolism Assay. Tertiary hamster embryo cell cultures (10^6 cells per 25-cm^2 flask, 3 flasks per group) were plated in 8 ml of medium containing 10% calf serum and refed with 8 ml of fresh medium after 48 h. Seventy-two h after plating, the cultures were treated with the test compound in DMSO or DMSO as a control, and 30 min later [^{3}H]-B(a)P (1 μg/ml; specific radioactivity, 0.25 Ci/mmol) was added. Twenty-four h later medium was removed and stored at −20°C. Aliquots (0.2 ml) were extracted by a two-stage chloroform:methanol:water procedure (6, 9). The assay uses initial mixing with a vortex mixer in a single-phase system of chloroform:methanol:water (including the medium) (1:2:0.8) to ensure complete extraction of the lipophilic hydrocarbon and its metabolites followed by addition of 1 ml of chloroform

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[3] The abbreviations used are: B(a)P, benzo(*a*)pyrene; HPLC, high-performance liquid chromatography; TLC, thin-layer chromatography; NMR, nuclear magnetic resonance; MS, mass spectrum; BHA, butylated hydroxyanisole; DMSO, dimethyl sulfoxide; B(a)PDE, 7,8-dihydroxy-9,10-epoxy-7,8,9,10-tetrahydrobenzo(*a*)pyrene; dGuo, deoxyguanosine.

and 1 ml of water and mixing with a vortex mixer. After centrifugation for 10 min, the aqueous phase was removed and extracted with 2.0 ml of chloroform to ensure complete extraction. The chloroform extracts were then pooled, and the radioactivity in the organic and aqueous-methanol phases was measured by liquid scintillation counting of 0.1-ml aliquots. This extraction procedure results in recovery of unmetabolized B(a)P and Phase I metabolites (dihydrodiols, quinones, and phenols) in the chloroform phase. The water-soluble metabolites including glucuronides and glutathione conjugates and multiple oxidation products are retained in the aqueous-methanol phase. Since the large majority of the metabolites formed from B(a)P in hamster embryo cells are water soluble (usually greater than 80%) (6), this assay provides a rapid measure of B(a)P metabolism.

BHA, a known inhibitor of carcinogenesis and B(a)P metabolism (10), was used to treat a positive control group in all assays at a concentration of 50 μg/ml of medium. The highest nontoxic dose of BHA was selected from multiple experiments using different hamster embryo cell preparations. Doses of 75, 65, 50, and 5 μg/ml of medium were tested, and the results show that 75 μg/ml were toxic and 65 μg/ml exhibited borderline toxicity, while 50 μg/ml showed a significant inhibition of B(a)P metabolism with no cell toxicity. The lowest dose, 5 μg/ml, produced no significant inhibition of B(a)P metabolism. Using BHA as a positive control gave us an indication of the health and viability of the cells in the culture assay for that particular experiment and helped eliminate false negatives.

Analysis of B(a)P Metabolites. The B(a)P metabolites in the organic phase were analyzed by HPLC on an Ultrasphere C_{18} column (25 cm × 4.6 mm) eluted with a methanol:water gradient as described previously (6). UV absorbing standards of authentic B(a)P metabolites (Chemical Repository, Division of Cancer Etiology, National Cancer Institute) were included in each HPLC analysis. The radioactivity was monitored with a Flo-one β flow monitor set to update every 30 s.

Binding of B(a)P to DNA. Tertiary hamster embryo cell cultures (5×10^7 cells) were plated in 175-cm^2 flasks containing 50 ml of minimal essential medium with 10% fetal bovine serum. After 2 days the cultures were refed with fresh medium and 24 h later with the test compound, or extract in DMSO was added. Five to 10 min later the cultures were treated with [^{3}H]B(a)P (1 μg/ml of medium, 0.5 mCi/flask). After 24 h of incubation at 37°C the cells were harvested, and DNA was isolated as described previously (11). The radioactivity in an aliquot was measured by liquid scintillation counting, the amount of DNA was determined by A_{260}, and these values were used to calculate the level of B(a)P metabolites bound to DNA.

After enzymatic degradation of the DNA to deoxyribonucleosides, the B(a)P:deoxyribonucleoside adducts were isolated by chromatography on Sep-Pak C_{18} cartridges and analyzed by HPLC on a 25-cm × 4.6-mm Ultrasphere C_8 reversed-phase column (11). The column was eluted at a flow rate of 1.0 ml/min with methanol:water (46:54) for 34 min, a linear gradient for 10 min (46:54 to 55:45) and at 55:45 for 24 min. Fifteen 1.0-ml fractions followed by 165 fractions (0.3 ml) were analyzed by scintillation counting.

Plant Extraction. Leaves, stems, and flowers of *Trifolium pratense* L (red clover) were collected. A voucher specimen is on deposit in the biology herbarium of the Department of Biology, Purdue University. The fresh plant (918 g) was ground with 2 liters of 95% ethyl alcohol in a commercial size Waring blender for 5 min. The blended material was then allowed to stand for 30 min to complete the extraction. The material was then filtered through a Büchner funnel, and the filtrate was concentrated *in vacuo* to give 46.5 g of the 95% ethyl alcohol extract. An aliquot was dissolved in DMSO and submitted for testing. The 95% ethyl alcohol extract was found to be active and therefore was then further partitioned according to the scheme shown in Fig. 1. The testing data are shown in Table 1. All fractions were tested at the dose-response dose which was defined as the percentage of the 95% ethanol-extractable material that the fraction represented times the dose of 95% ethanol fraction used in the metabolism assay (in this case, 750 μg/ml of medium).

Isolation and Identification of Active Components. Aliquots from the solvent partition were submitted for testing. The active $CHCl_3$ fraction was subjected to silica gel flash column chromatography with hexane, $CHCl_3$, ethyl acetate, acetone, acetone:methyl alcohol (1:1), and finally methyl alcohol. Nine fractions were collected, and aliquots were taken and submitted for testing. The column fraction which was active at the dose-response dose (Fraction 1D) (see Fig. 1) was further chromatographed by centrifugal silica gel TLC (Chromatotron) using a $CHCl_3$/methyl alcohol solvent gradient starting with 2% methyl alcohol in $CHCl_3$. The fractions which were collected were combined according to the presence of similar spots when analyzed by silica gel TLC developed in 2% methyl alcohol in $CHCl_3$. Based upon this, the samples were combined into seven fractions which were tested for their effects on B(a)P metabolism. The most active fraction (2D) was further separated on another silica gel Chromatotron plate developed in a $CHCl_3$/methyl alcohol gradient. Based upon TLC profiles eluants were combined into three fractions. The most active fraction (3B) contained a major component. Recrystallization of this fraction from aqueous methyl alcohol gave a crystalline material, m.p. 217–218°C. A sample of authentic biochanin A was purchased from Aldrich Chemical Co., m.p. 218–219°C. A mixed m.p. showed no depression. The UV and ^{1}H NMR data were identical to literature values (12), and the MS and ^{13}C NMR data were consistent with the published structure.

Examination of the interface fraction, which was active at 2× the dose-response dose, led to the isolation of additional biochanin A, along with an analogue, formononetin (see Fig. 3). Formononetin was inactive in the metabolism bioassay. Biochanin A represented about 30% of the interface fraction.

RESULTS

The results of bioasssay-directed fractionation of the active ethyl alcohol extract of red clover are presented in Fig. 1 and Table 1. The ethyl alcohol extract was active at doses from 500 μg/ml to 1000 μg/ml; however, toxicity was detected at the highest dose (see Table 1). Further partitioning of the active ethyl alcohol extract was dose responded from 750 μg/ml. After partitioning between chloroform and water, the activity appeared in the chloroform extract. Examination of the interface which was active at twice the dose-response dose confirmed the presence of biochanin A. Chromatography of the chloroform fraction gave active column Fraction 1D. This fraction was carried through two separations on the Chromatotron to give in turn active Fractions 2D an 3B. Crystallization of Fraction 3B gave 30 mg of the active constituent, biochanin A. Fractions 1A, 1B, 2B, and 2F show activity at twice the dose-response dose and are under further investigation. The B(a)P metabolites present in the organic phase of the sample treated with red clover extract at 500 μg/ml were analyzed by HPLC, and the amount of the major primary B(a)P metabolites was determined (Fig. 2). The two major changes were a slight increase in the amount of 9-hydroxy-B(a)P and a major decrease in the amount of water-soluble metabolites in the extract-treated group. After β-glucuronidase treatment of the aqueous phase, the amount of 9-hydroxy- and 3-hydroxy-B(a)P in the red clover extract-treated group was reduced by 30% and 22%, respectively, when compared to DMSO controls. The water-soluble metabolites were also decreased by 18% in the red clover extract-treated cells. Thus the major effect of red clover extract was to inhibit the formation of B(a)P-phenol glucuronides.

The effect of the crude 95% ethyl alcohol extract on the binding of B(a)P to DNA was also examined (Table 2). At a dose of 250 μg/ml the extract inhibited B(a)P-DNA binding by 30% to 41% comparred to controls in three separate experiments. Analysis of the B(a)P-DNA adducts present in enzyme-digested DNA samples by HPLC demonstrated that the extract inhibited the formation of both the *syn*- and *anti*-isomers of B(a)PDE. The *syn*-B(a)PDE-dGuo adducts decreased from 37% to 64% compared to controls, and the (+)-*anti*-B(a)PDE-dGuo adduct decreased from 48% to 75%.

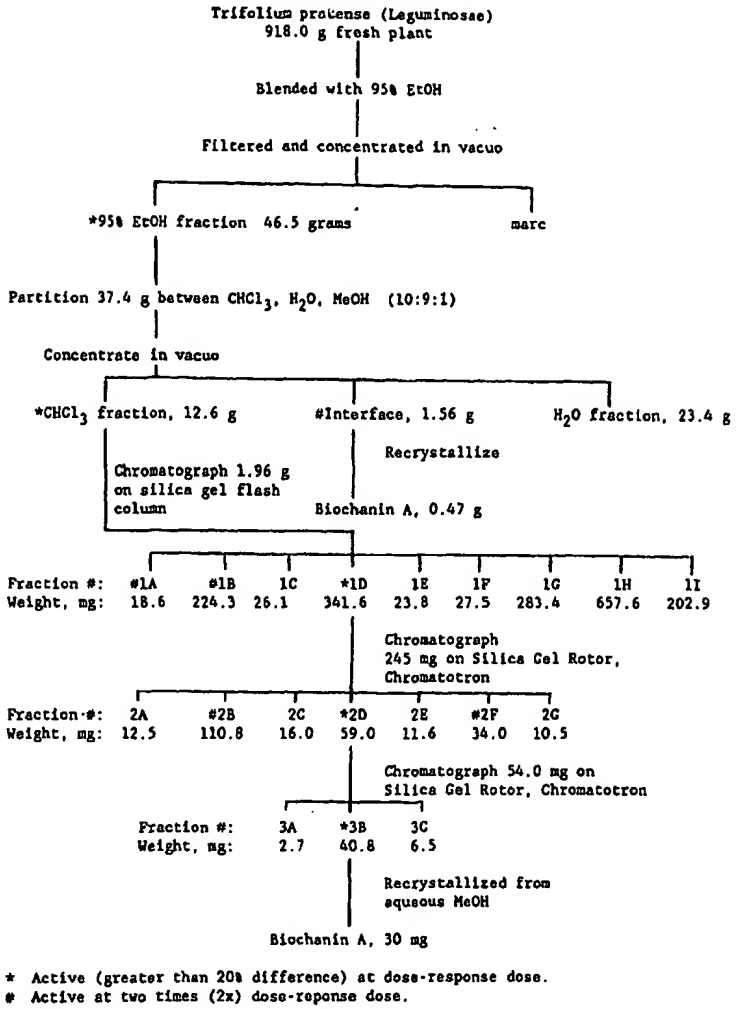

Fig. 1. Fractionation scheme for the 95% ethyl alcohol extract of fresh red clover leaves, flowers, and stems. *EtOH*, ethyl alcohol; *MeOH*, methyl alcohol.

The effect of biochanin A on the binding of B(a)P to DNA in hamster embryo cell cultures was also examined. Biochanin A caused a 54% decrease in B(a)P metabolism at 25 μg/ml. After exposure of cultures to 25 μg of biochanin A and 1 μg of [^{3}H]B(a)P per ml of medium for 24 h, biochanin A treatment reduced the amount of B(a)P bound per mg of DNA from 74.3 pmol in the control group to 35.1 pmol in the biochanin A group in one experiment and from 72.2 to 45.4 in a second experiment. Thus, biochanin A inhibited the binding of B(a)P to DNA to an extent similar to that obtained in the crude extract (Table 2).

DISCUSSION AND CONCLUSIONS

There are several bioassays which are under investigation for the detection of compounds suspected of having potential cancer chemopreventive activity. Antimutagenic activity in the form of an anti-Ames assay has been commonly used in the United States and Japan (13, 14). Mitscher *et al.* (15) used this bioassay to isolate and identify glabrene, a known isoflavene exhibiting antimutagenic activity. Nishino *et al.* (16) used the antitumor-promoting activity of glycyrrhetic acid against 7,12-dimethylbenz(a)anthracene and teleocidin as a model of cancer prevention. The decrease in formation of carcinogenic *N*-nitroso compounds produced by α-tocopherol and ascorbic acid was used as a criterion for chemoprevention by Narkus *et al.* (17) and Mervish (18). Sakiyama *et al.* (19) used the inhibition of transformation of the mouse 10T½ cell line induced by X-ray or *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine as a model to show the anticarcinogenic effects of lipopolysaccharides and indomethacin. The induction of aryl hydrocarbon hydroxylase activity in liver and intestinal mucosa of Sprague-Dawley rats was used by Wattenberg *et al.* (3) to isolate and identify a group of indoles from cruciferous vegetables (20, 21). Another screen by Wattenberg *et al.* (5) used the induction of glutathione *S*-transferase activity, a major detoxification enzyme system, for a number of electrophiles, including many carcinogens, in mouse liver and intestinal mucosa to isolate a group of known diterpenes from green coffee beans.

The screening procedure described in this paper measures effects on the ability of hamster embryo cell cultures to metabolize the carcinogen B(a)P. Induction of inhibition of B(a)P metabolism of treated cultures by >20% as compared with control cultures was considered to be an active test. The altered pattern of metabolism was determined by HPLC analysis of the B(a)P metabolites formed, and the effects on binding of B(a)P to DNA are determined. Confirmed active extracts are then fractionated using the bioassay as a guide. Advantages of our method are that activity data can be generated within a few days after the extract or compound is tested, and a large number

Table 1 *Activity of fractions in B(a)P metabolism assay*

The procedure used for analysis of B(a)P metabolism to water-soluble metabolites is described in "Materials and Methods."

Fraction	Dose-response dose (μg/ml medium)	% of change from control	2× dose-response dose	% of change from control
95% Ethyl alcohol	500	−39.9 ± 6.2[a, b]		
	750	−40.4 ± 6.9[b, c]		
$CHCl_3$	203	−35.6 ± 5.7[b, c]	406	−68 ± 4.4[c]
Interface	25.2	−17.7 ± 7.3[c]	50.2	−33.8[d, e]
H_2O	541	−15.4 ± 1.4[c]		
1A	1.9	17.2 ± 5.4[a]	3.9	28.8[d, e]
1B	23.3	−5.9 ± 15.2[a]	46.6	−34.6 ± 4.6[c, e]
1C	3.7	8.7 ± 5.6[a]	7.4	−8.0[d]
1D	35.5	23.5 ± 4.0[b, c]	71.0	−50.2[d, e]
1E	2.5	3.25 ± 6.8[c]	5.0	−3.5[d]
1F	2.8	8.5 ± 0.8[c]	5.6	13.6[d]
1G	29.4	12.1 ± 7.3[c]	58.8	17.8[d]
1H	68.1	5.0 ± 9.2[c]	136.2	−5.5[d]
1I	21.0	−5.9 ± 11.4[c]	42.0	−11.7[d]
2A	1.8	−14.1[d]	3.6	4.8[d]
2B	16.1	−12.9[d]	32.2	−32.7[d, e]
2C	2.3	−13.8[d]	4.6	−12.9[d]
2D	8.6	−25.3[b, d]	17.2	−19.9[d]
2E	1.7	−13.0[d]	2.4	3.0[d]
2F	4.9	−14.6[d]	9.8	−23.0[d, e]
2G	1.5	−1.8[d]	3.0	
3A	0.4	−6.8[d]	0.8	
3B	6.4	−23.7[b, d]	12.8	−23.0 ± 22.9[e, f]
3C	1.0	5.93 ± 18.6[a]	2.0	8.8[d]
Biochanin A	4.7	−12.2[d]		
	9.5	−32.1[d]		
	19.0	−47.4[d]		
	23.6	−48.8[d]		

[a] Mean ± SD of 3 experiments.
[b] Active (greater than 20% difference) at dose-response dose.
[c] Average ± range of 2 experiments.
[d] One experiment.
[e] Active at 2× dose-response dose.
[f] Mean ± SD of 4 experiments.

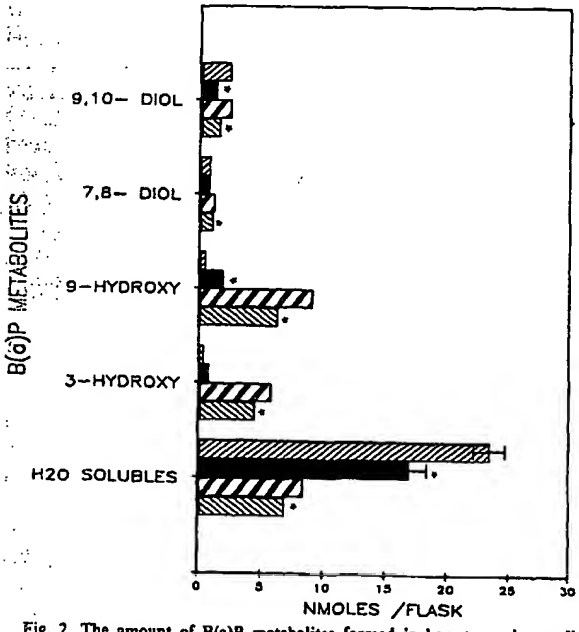

Fig. 2. The amount of B(a)P metabolites formed in hamster embryo cell cultures in the presence or absence of 500 μg/ml of red clover crude extract. The cultures were treated, and the medium samples used analyzed as described in "Materials and Methods." Medium samples were treated with β-glucuronidase prior to extraction to determine glucuronide conjugates. *Columns*, mean for 3 flasks per group; *bars*, SD. *, red clover extract-treated samples that differed significantly from the corresponding control (based upon Student's *t* test; $P < 0.01$). ▨, control; ■, *T. pratense*; ▨, control (β-Glucuronidase); ▩, *T. pratense* (β-Glucuronidase).

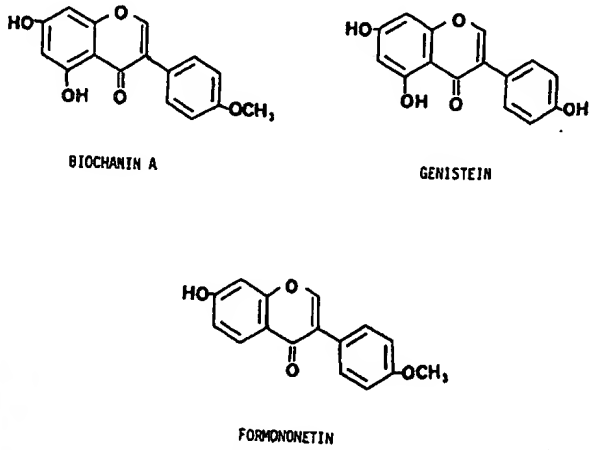

Fig. 3. Isoflavones isolated from red clover leaves and flowers (25, 26).

of different samples can be screened simultaneously. After pure active compounds are isolated and their effect on the metabolic activation of B(a)P is established, they will then be further tested using *in vivo* bioassays to determine their effect on tumor induction by various classes of carcinogens. These *in vivo* bioassays are essential for determining whether a compound acts as an anticarcinogen and against which classes of carcinogens it was active.

Thus far we have screened over 70 species and varieties of plants and vegetables comprising 27 families. One of the first plant extracts demonstrated to produce reproducible inhibition of B(a)P metabolism in the hamster cell culture assay was that prepared from red clover. Based upon inhibition of B(a)P metabolism the crude red clover extract was fractionated, and

Table 2 *B(a)P-DNA binding in hamster embryo cells treated with extracts of red clover*

Hamster embryo cell cultures were exposed to the 95% ethyl alcohol extract of red clover at a dose of 250 μg/ml of medium, and 10 min later 1.0 μg of [^{3}H]B(a)P per ml of medium was added. After 24 h, the medium was removed, and a portion was analyzed by chloroform:methanol extraction as described in "Materials and Methods." The percentage of radioactivity in the water phase is reported as the percentage of water-soluble B(a)P metabolites. The DNA was isolated from the cells, and the level of binding of B(a)P was measured. The DNA was digested to deoxyribonucleosides, and the amount of the major B(a)P-DNA adducts was determined by HPLC.

	Experiment 1		Experiment 2		Experiment 3	
	Control	Test extract	Control	Test extract	Control	Test extract
% of water-soluble B(a)P metabolites	48.4	40.5	30.8	15.4	35.6	27.5
Binding of B(a)P to DNA; total level of binding (pmol/mg DNA)	67.0	42.7	51.2	30.0	23.0	15.8
(+)-*anti*-B(a)PDE-dGuo adduct (pmol/mg)	12.7	4.9	11.1	2.7	5.5	2.9
B(a)PDE-dGuo adduct (pmol/mg)	8.4	3.0	8.1	3.7	5.0	3.3

the active compound, biochanin A, was isolated which produced an inhibition of B(a)P metabolism of 30 to 50% at doses of 23.6 μg/ml compared to DMSO controls. Exposure of hamster embryo cell cultures to biochanin A at a dose of 25 μg/ml of medium resulted in a 37 to 50% inhibition in the binding of B(a)P to DNA. This compound appears to be one of the major components responsible for the inhibition of B(a)P-DNA interactions by the red clover extract. The strong correlation between the binding of aromatic hydrocarbons to DNA and their carcinogenic activity suggests that biochanin A is a good candidate for further testing to measure inhibition of tumor induction by hydrocarbons in animals.

Several flavonoids have been shown to possess anticarcinogenic activity (3). 7,8-Benzoflavone, a synthetic flavonoid, is an inhibitor of microsomal mixed-function oxidases and inhibits the metabolism, binding to DNA, and tumorigenesis of 7,12-dimethylbenz(*a*)anthracene in mouse skin (22). This same flavonoid also inhibits the metabolism of B(a)P in rat hepatic microsomes that have been induced with 3-methylcholanthrene (23). Huang *et al.* (24) examined 28 flavonoids for their effect on the mutagenicity of anti-B(a)PDE in *Salmonella* and found that they had significant antimutagenic activity. Interestingly one of the flavonoids found to be inactive (50% inhibitory dose > 100) was genistein, an isoflavone related to biochanin A (see Fig. 3) and a minor constituent of red clover (25, 26). Since the compound tested [B(a)PDE] was an ultimate mutagenic metabolites of B(a)P, that assay would not be expected to detect compounds that alter metabolic activation of B(a)P. Thus, various types of short-term assays may be anticipated to detect anticarcinogens that work by different mechanisms. In view of the requirement of the majority of classes of chemical carcinogens for metabolic activation and the ability of the metabolism assay to measure changes in enzymes both involved in activation as well as detoxification, the hamster cell assay should be capable of detecting modifiers of carcinogen metabolism that work by a number of mechanisms. The results demonstrate that the effects of test compounds on B(a)P metabolism and DNA binding in hamster embryo cell cultures can be used to screen and isolate pure compounds with potential anticarcinogenic activity from plants and other natural products.

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SHORT PAPER

OESTROGENIC ACTIVITY OF SOYA-BEAN PRODUCTS

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Abstract—Normal rat cake containing soya meal was found to be oestrogenic. Sixteen samples of soya meal were examined in the mouse uterine weight bioassay and all were found to have oestrogenic activity. Ethyl-acetate extracts of the meals also had oestrogenic activity. Genistein and daidzein were present in the extracts.

Introduction

It has previously been reported from this laboratory (Drane, Patterson, Roberts & Saba, 1975) that rat cake used as a control feed in routine mouse bioassays for oestrogens had developed significant uterotrophic activity over a period of a few months. We have recently encountered another oestrogenic control feed, higher basal uterine weights than expected being found in mice fed this ration. Investigation of the components of the feed showed that the oestrogenic activity was due to soya meal, which made up 10% of the rat cubes.

Little attention seems to have been paid to soya meal as a possible source of oestrogenicity although daidzein and genistein were isolated from soya beans nearly 50 yr ago (Walz, 1931). The oestrogenic activity of these and other isoflavones is well documented (Bickoff, Livingston, Hendrickson & Booth, 1962; Carter, Smart & Matrone, 1953; Cheng, Story, Yoder, Hale & Burroughs, 1953). A new isoflavone, glycitein, has been isolated from soya beans (Naim, Gestetner, Kirson, Burk & Bondi, 1973) and recently coumestrol was also shown to be present at levels ranging from 0·05–30 μg/g (Lockhart, Jones & Finney, 1978). The present report provides bioassay data showing that oestrogenic activity was present in all sixteen samples of soya meal examined.

Experimental

Materials. Sample 1 was the extracted soya-bean meal that had been used in the manufacture of the control feed (Porton Rat Diet) associated with the original problem. Samples 2–14 were soya-bean meals of various origins destined for the manufacture of farm-animal feeds. Sample 11 was a pelleted form of feed. Samples 15 and 16 were soya-bean products intended for human consumption. Semi-synthetic (SS) feed supplied by RHM Labsure Ltd. was used as a soya-free control.

Three extracts were prepared. For the first, 90 g soya-bean meal was exhaustively extracted with ethyl acetate in a Soxhlet apparatus. The solvent was evaporated to dryness and the residue was re-dissolved in a convenient volume of ethanol–ethyl acetate (1:1, v/v). A second, 70%-ethanol extract was prepared as described for the extraction of oestrogens from white clover (Saba, Drane, Hebert & Holdsworth, 1974). A third extract in aqueous acetonitrile was also prepared (Drane *et al.* 1975).

Oestrogen bioassay. Eighteen-day-old MF1 weanling female mice weighing 7–9 g were supplied by OLAC 1976 Ltd., Bicester, Oxon. They were housed in groups of six to a cage and each group was given 40 g of feed over a period of 3·5 days. On the following day the mice were killed and the uteri were dissected out, blotted on filter paper and weighed. Each assay included a control group given only the SS feed and three or four groups given SS feed containing known amounts of diethylstilboestrol (DES). The test soya meal samples were fed alone, or mixed with SS diet, or as an extract mixed into SS diet and air dried.

Mycology and mycotoxin screening. Samples of soya-bean meal were screened for possible mycotoxin contamination by the method described by Roberts & Patterson (1975) as modified by Patterson & Roberts (1979). The mycological examination of six samples was carried out by the methods described by Shreeve, Patterson & Roberts (1975).

Thin-layer chromatography (TLC). Biologically-active ethyl-acetate extracts were examined for phyto-oestrogens by TLC using Polygram Sil G/UV_{254} sheets and methanol–chloroform (7:93, v/v) as the developing solvent. Genistein, daidzein and formononetin (minimum detectable levels 10 μg/g) and coumestrol (minimum detectable level 1 μg/g) were run as reference compounds. The developed chromatogram was examined under long- (360 nm) and shortwave (250 nm) ultra-violet light for fluorescing and absorbing spots both before and after exposure to ammonia vapour. These active extracts were also analysed for zearalenone using the mycotoxin method cited above (analytical limit 20 μg/kg).

Results and Discussion

No mould growth was evident in any of the six soya meals sampled and Fusarium species were not

isolated in mycological cultures of the meals. Neither zearalenone nor any other mycotoxin was detected.

The mouse uterine weight bioassay data are summarized in Table 1. Samples 2 and 3 were oestrogenic when fed as whole meal but their extracts were not tested. Nine other samples were active when fed as whole meal and also when fed in the form of ethyl-acetate extracts, while five further samples were active only when fed as ethyl-acetate extracts. Thus all sixteen samples showed biological activity. No oestrogenic activity was found in extracts in 70% aqueous ethanol, which is routinely used to extract substances with oestrogenic activity from red and white clover (Bickoff, Loper, Hanson, Graham, Witt & Spencer, 1967; Saba *et al.* 1974). Neither was it found in extracts in acetonitrile, which has previously been used to isolate an active fraction 6bII from oestrogenic rat cake (Drane *et al.* 1975). However, all of the ethyl-acetate extracts were oestrogenically active, although when appropriate comparisons were made, it was found that the recovery of the source of the activity present in the original samples of soya meal was poor. Hydrolysed ethyl-acetate extracts examined by TLC were found to contain genistein and daidzein and preliminary experiments suggested that the former isoflavone contributed most of the oestrogenic activity. No other reference oestrogen was detected.

Various reproductive disturbances in animals have been traced to the ingestion of oestrogenic feeds. Cattle became infertile whilst grazing lucerne containing high concentrations of coumestrol (Adler & Trainin, 1967), hyperoestrogenism was reported in pigs fed diets containing 0·1–6·8 μg zearalenone/g (Mirocha, Pathre & Christensen, 1977), the conception rate was lowered in sheep fed 8–16 μg DES/day and conception was prevented altogether in sheep given 32 μg DES/day (Morley, Bennett & Axelsen, 1963). The present results suggest that comparable levels of oestrogenic activity might be provided by diets containing soya products; in those whole soya meals in which quantifiable amounts of oestrogenic activity were present, levels equivalent to 8–37 ng DES/g soya were detected. On the basis of our own estimate that the potency of the mycotoxin zearalenone in the mouse bioassay is $8{\cdot}5 \times 10^{-4}$ that of DES, the observed oestrogenic activity of these soya meals was equivalent to 9·4–43·3 μg zearalenone/g soya.

There is little published information on the oestro-

Table 1. *Oestrogenic activity of whole soya meal and of ethyl-acetate extracts of whole soya meal in the mouse uterine weight assay*

	Results for mice fed whole soya meal				Results for mice fed ethyl-acetate extracts of soya meal			
Sample no.	Maximum total dose† (g whole soya meal/mouse)	Uterine wt (geometric mean; mg)	DES equivalent (ng/g soya)	Calculated zearalenone equivalent‡ (μg/g soya)	Maximum total dose† (g soya meal extracted/ mouse)	Uterine wt (geometric mean; mg)	DES equivalent (ng/g)	Calculated zearalenone equivalent‡ (μg/g soya)
1	6	10·4*	<10§		NT			
	6	16·5**	8	9·4	15	19·4***	5	5·9
2	6	11·7	<10		NT			
	6	13·9*	17	19·9	NT			
3	6	16·0***	10–20		NT			
	6	17·4***	17	19·9	NT			
	6	19·0***	10	11·7	NT			
4	6	11·3***	<20		NT			
	6	13·6***	10	11·7	20	18·6***	3·5	4·1
5	3	12·2**	<10		15	16·6***	4	4·7
6	3	8·5	0·0		15	19·0***	5	5·9
7	3	11·0*	<10		14	33·5***	8	9·4
8	2·5	6·3	0·0		12	29·1***	7	8·2
	5	10·5	<10		NT			
9	2·5	7·7	0·0		14	21·3***	4	4·7
	5	9·0	<10		NT			
10	2·5	10·1**	<10		12	24·0***	5	5·9
11	3	7·8	0·0		12	15·5***	3	3·5
12	2·5	9·9	<10		12	22·8***	5	5·9
13	3	31·5***	37	43·3	15	56·5***	12	14·0
14	3	13·6***	12	14·0	15	32·7***	7	8·2
15	5	34·0***	24	28·1	15	36·3***	8·7	10·2
16	4	15·5***	16	18·7	15	25·3***	7	8·2

NT = Not tested

†The mice ate poorly, and therefore the dose is only approximate.

‡By the mouse uterine weight assay the zearalenone equivalent per unit wt of DES = 1170 (850–1600).

§Oestrogenic activity present at levels equivalent to <10 ng DES/g soya could not be quantified.

The values marked with asterisks differ significantly from those of the controls that were given soya-free semi-synthetic feed (*$P < 0{\cdot}05$; **$P < 0{\cdot}01$; ***$P < 0{\cdot}001$). Sample 1 was a soya-bean meal used in the manufacture of rat feed. Samples 2–14 were soya-bean meals used for the manufacture of farm-animal feeds. Samples 15 and 16 were soya-bean products intended for human consumption.

genic activity of foodstuffs for human consumption (Schoental, 1977) and this report helps to remedy the situation. Since soya meal is an important source of protein for animal feeds and is now increasingly used in human food, we feel that this apparently constant source of oestrogenic activity should not be overlooked, even though it is at a low level. However, species differ greatly in their susceptibility to the effects of oestrogens and caution must therefore be exercised when attempting to extrapolate data from species to species or from the biological effects of one oestrogenic substance to another.

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E6

Oestrogenwirksame Isoflavone in Trifolium pratense (Rotklee)

Verteilung in den oberirdischen Pflanzenteilen und Vorkommen als „gebundene" Isoflavone*)

Von G. Schultz — Mit 3 Abbildungen
Aus dem Botanischen Institut der Tierärztlichen Hochschule Hannover
Direktor: Professor Dr. E. Perner

Einleitung

Durch die Arbeiten von Bennetts (1946) wurde bekannt, daß in Australien bei Schafen, die auf Trifolium subterraneum geweidet wurden, gehäuft Fertilitätsstörungen auftraten. Ähnliche Beobachtungen konnten auch in anderen Gebieten der Erde gemacht werden (siehe Zusammenfassung von Moule, Braden und Lamond 1963). Weitere Untersuchungen ergaben, daß diese Störungen mit oestrogenwirksamen Vertretern aus der Gruppe der Isoflavonen in Zusammenhang stehen. Es handelt sich um Inhaltsstoffe, deren Vorkommen in Leguminosen schon längere Zeit bekannt sind (Walz 1931).

So wurde in Trifolium subterraneum Genistein (Bradbury und White 1951) und Biochanin A (Pope und Mitarbeiter 1953) als oestrogenwirksame Isoflavone erkannt. In Trifolium repens (Varietät Ladino) und in Luzerne (Medicago sativa) konnte durch die Arbeitsgruppe von Bickoff 1957 als wirksame Substanz das von den Isoflavonen biogenetisch abgeleitete (Grisebach 1964) Cumöstrol erkannt werden.

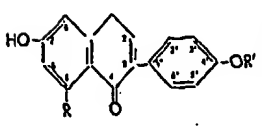
Von den in Mitteleuropa vorhandenen Kleearten interessiert jedoch in erster Linie Trifolium pratense (Rotklee). Hierüber hat allein Wong 1963 den Isoflavonengehalt von einigen in Neuseeland angebauten Sorten (N. Z. Montgomery, N. Z. Broad red, Tetraploid und Moroccan) untersucht.

Für die eigenen Untersuchungen wurde die in Norddeutschland vornehmlich angebaute Sorte Original Lembke verwendet.

Nachdem quantitative Angaben über die Verteilung der Isoflavone in verschiedenen Teilen der Pflanze fehlen, wurde der Isoflavon-Gehalt in Blattspreite, Blattstiel und Sproßmaterial vergleichend untersucht und es war zu prüfen, wie sich der Gehalt der wirksamen Isoflavonen Biochanin A und Formononetin im Verlauf der Vegetationsperiode ändert.

Vorversuche ergaben, daß nach erschöpfender Extraktion des Blattmaterials mit Alkohol aus dem Rückstand mit halbkonzentrierter Salzsäure weitere Isoflavone freigesetzt werden konnten. Damit war anzunehmen, daß diese hauptsächlich in gebundener Form vorliegen und quantitativ erst nach der Hydrolyse zu erfassen sind.

*) Bei den hier aufgeführten Isoflavonen handelt es sich um Genistein (= 5, 7, 4' — Trihydroxy-Isoflavon), Biochanin A (= 5, 7-Dihydroxy-4'-Methoxy-Isoflavon), Daidzein (= 7, 4'-Dihydroxy-Isoflavon) und Formononetin (= 7-Hydroxy-4'-Methoxy-Isoflavon).



R = OH R' = H Genistein
R = OH R' = CH_3 Biochanin A
R = H R' = H Daidzein
R = H R' = CH_3 Formononetin

Diese Befunde stimmen mit Beck 1964**) überein, der zeigen konnte, daß Isoflavone durch ein hydrolytisch wirkendes Ferment in der Blattspreite freigesetzt werden und sich erst dann mit Alkohol extrahieren lassen. Das Ferment wird aber erst nach der Zerstörung der Pflanzenzellen wirksam. Eine Inaktivierung des Ferments durch Alkohol oder Erhitzung führt zu unvollständiger Extraktion der gebundenen Isoflavone. Diese kann teilweise durch Inkubation des homogenisierten Kleematerials mit β-Glucosidase und damit Freisetzung der Isoflavone erreicht werden.

In dem Versuch wurde auch Lolium perenne (Deutsches Weidelgras, Original Lembke) als wichtigstes mitteleuropäisches Weidelgras einbezogen, nachdem Schoop und Klette 1955 Angaben über das Vorkommen von Oestrogenen in „Koppelgras" machen konnten. Es ergab sich jedoch, daß Weidelgras keines der bekannten Isoflavone enthält.

Methodik

Versuchsanlage: Rotklee Original Lembke aus deutschen Eliten wurde im Mai 1964 auf schwach humosem Sandboden im Botanischen Institut der hiesigen Hochschule ausgesät. Die Anlage war ursprünglich als Düngungsversuch in 19 Parzellen zu je 5×1 m geplant. Eine dahingehende Auswertung war aber nach den Ergebnissen der Bodenuntersuchung*) nicht möglich, da vermutlich infolge schwacher Sorption des Bodens die errechneten Unterschiede im Nährstoffgehalt nur schwach zum Vorschein kamen. Der Boden wies bezüglich der Stickstoffversorgung einen spürbaren Mangel auf. Der Phosphatgehalt war normal, Kali war im Überschuß vorhanden.

Probennahme: 2 Teilstücke jeder Parzelle zu 0,35×1,0 m wurden gemäht und die geerntete Frischmasse gewogen. Im ersten Versuch wurden davon je 50 g pro Parzelle, in den späteren Versuchen bis zu 200 g Frischgewicht für die Bestimmung des Isoflavon- und Trockensubstanzgehalts entnommen. Diese Proben wurden anfänglich mit siedendem Alkohol übergossen, im Starmix homogenisiert und im Trockenschrank bei 80° C bis zur Gewichtskonstanz getrocknet. Dabei ergab sich bei Formononetin ein durchschnittlicher Verlust von 8,5 ± 5 %, bei Biochanin A von 29 ± 3 % gegenüber Trockensubstanz, die über nur kurze Zeit im Trockenschrank bei 70—80° getrocknet wurden.

Extraktion: Die Extraktion erfolgte im wesentlichen nach Wong 1962, jedoch wurden für die quantitativen Serienbestimmungen einige Vereinfachungen eingeführt, die die Analysengenauigkeit nicht wesentlich beeinflussen. Auf die Beseitigung von Lipiden, Chlorophyll sowie dessen Abbauprodukte durch Ausschütteln der Extrakte mit Petroläther wurde verzichtet. Zur Chromatographie wurden äußerst geringe Substanzmengen aufgetragen, so daß damit die gegenseitige Störung vermieden wurde. Zum Unterschied von den bisherigen Verfahren wurde zu der in Alkohol suspendierten Trockensubstanz im Laufe der Extraktion 0,7 — 1 Vol. konz. Salzsäure zur Hydrolyse der Isoflavonverbindungen zugesetzt (die Verluste belaufen sich auf etwa 10—15 %).

500 mg im Starmix gemahlener Trockensubstanz wurde mit 30 ml Alkohol 20 min unter Rückfluß gekocht. Zu der Suspension wurden 20 ml HCl d = 1,12 zugefügt und weitere 10 min unter Rückfluß gekocht. Nach Filtration durch ein G2-Nutsche und Waschung des Rückstandes mit heißem Alkohol wurde das Filtrat im Vakuum am Rotationsverdampfer zur Trockne eingeengt. Die braungrüne Substanz wurde mit warmem Alkohol gelöst und auf insgesamt 20 ml aufgefüllt.

Bestimmung: Die semiquantitative Bestimmung erfolgt dünnschichtchromatographisch in Anlehnung an

*) Die Bodenuntersuchung wurde vom Bodenuntersuchungs-Institut Koldingen bei Hannover (Herrn Schulze) nach der Methode von Prof. Tepe, Gelsenheim, durchgeführt.

Die Mittelwerte (und eingeklammert die Extremwerte) des Nährstoffgehalts in mg ausgetauscht pro 24 Std. pro 250 cm³ Boden betrugen:

K_2O in mg	7,6	(5,1 —10,5)	erwünschter	3 — 5 mg
Na_2O	1,0	(0,4 — 1,4)	Optimalbereich	1 — 3
CaO	7,0	(6,0 — 8,0)		15 — 40
MgO	1,1	(0,9 — 1,3)		1 — 3
Mn	0,05	(0,04 — 0,05)		0,05 — 0,5
P_2O_5	0,7	(0,5 — 1,1)		0,3 — 1,0
SO_4	0,3	(0,2 — 0,5)		1 — 4
N	0,7	(0,4 — 1,0)		1 — 5
pH in KCl	5,5	(5,2 — 5,9)		

**) Herr Professor Dr. H. Grisebach, Freiburg i. Br., machte mich freundlicherweise auf diese Arbeit aufmerksam.

Weiterhin bin ich Herrn Prof. Dr. Grisebach für die Überlassung von Proben Biochanin A, Daidzein und Formononetin zu großem Dank verpflichtet.

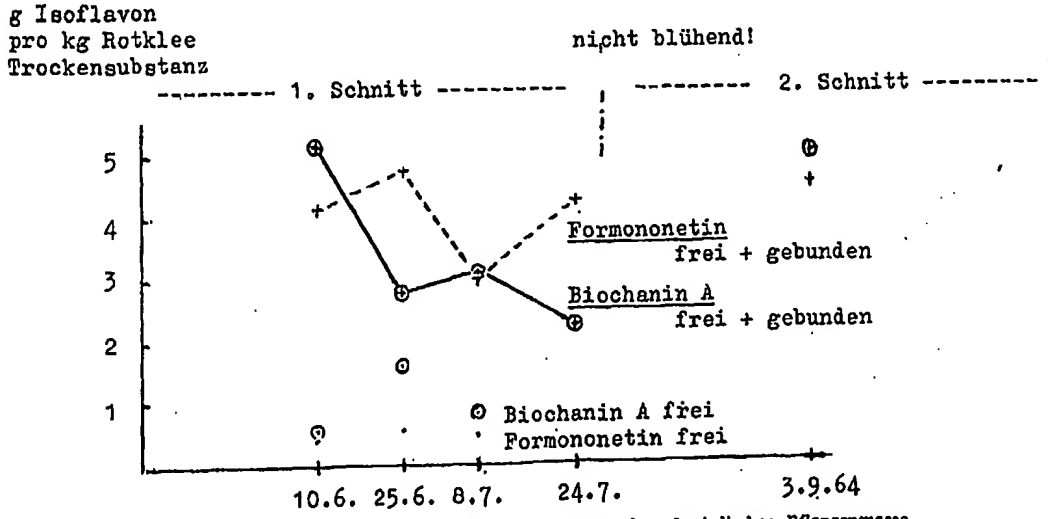


Abb. 1: Isoflavongehalt bezogen auf Trockensubstanz der oberirdischen Pflanzenmasse.

Beck 1964 durch Vergleich mit Eichsubstanzen. Als Träger diente Kieselgel G von Merck in 250 u Stärke auf 20X20 cm-Platten. Zur Verminderung der Absorption und zur Verbesserung der flächenförmigen Ausbreitung der getrennten Substanzen wurden die Platten in einem mäßig feuchten Raum luftgetrocknet. Aufgetragen wurden Mengen, die 0,25—2 % Formononetin und 0,5—2 y Biochanin A entsprachen. Jede Probe wurde in drei Konzentrationsstufen aufgetragen. Als Laufmittel diente Benzol/-Propanol 89:11 (von Deimling 1962), teilweise auch Benzol/8 Vol.-% Äthanol (Grisebach 1963). Zur Erkennung und zur visuellen Vergleichsbestimmung wurde beim Formononetin die starke blauweiße Fluoreszenz im UV-Licht ausgenutzt. Biochanin A wurde dagegen erst nach Besprühen mit diazotierter Sulfanilsäure (siehe Randerath 1962) an der braungelben Anfärbung sichtbar.

Ergebnisse

Abb. 1 zeigt den Gehalt von Formononetin und Biochanin A in der Trockenmasse der gesamten oberirdischen Pflanzenorgane im Laufe eines Sommers, wobei sich die Kurve auf Werte stützt, die nach vollständiger Hydrolyse mit Salzsäure vorlagen. Die in der Abb. 1 angegebenen Punkte nahe der Abzisse beziehen sich dagegen auf die freien Isoflavone, die sich mit heißem Alkohol ohne zusätzliche Hydrolyse nachweisen ließen. Auffällig sind die zum Teil beträchtlichen Unterschiede zwischen freien und gebundenen Isoflavonen.

Nach den im Abstand von etwa 14 Tagen entnommenen Proben des 1. Schnitts ergibt sich, daß der Biochanin-A-Gehalt im Laufe der Entwicklung der Pflanzen merklich abfällt, während der Formononetingehalt etwa konstant bleibt.

Die am 3. 9. geerntete Probe des 2. Aufwuchses entspricht ihrem Entwicklungszustand nach der Probe vom 10. 6. des 1. Aufwuchses. Auffällig ist der übereinstimmende Isoflavongehalt.

In Abb. 2 ist in den beiden linken Diagrammen der Gehalt von Formononetin bzw. Biochanin A von Blättern des vegetativen (gestauchten) Sprosses in zwei Altersstufen, im rechten Diagramm vom blühenden Sproß wiedergegeben: In der oberen Reihe stehen die Werte für die Blattspreite, in der unteren die für die Blattstiele bzw. für Blattstiel plus blühenden Sproß.

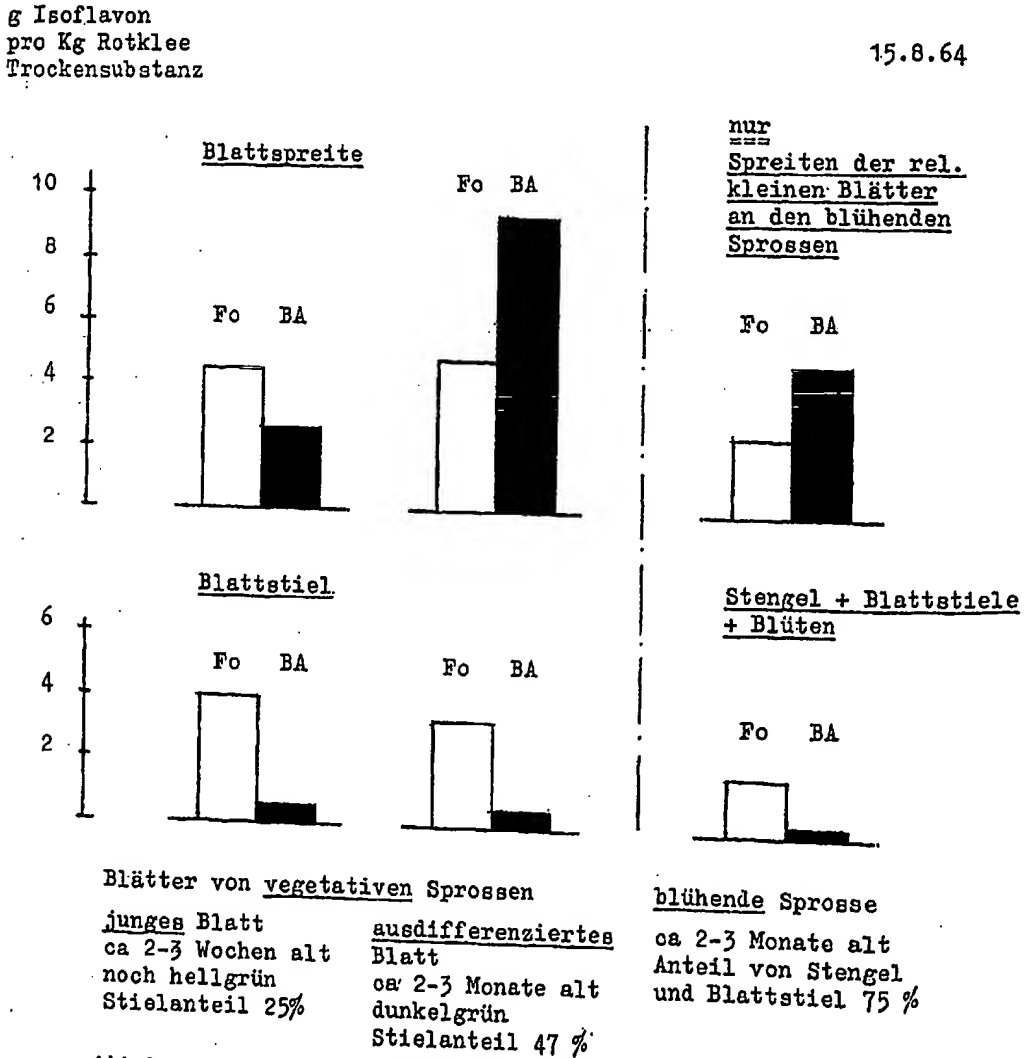


Abb. 2: Isoflavongehalt von Spreite (Blattfläche) und Stiel von Blättern verschiedenen Alters.

Danach ergeben sich bezüglich des Isoflavongehaltes charakteristische Unterschiede zwischen Blattspreite und -stiel. Generell ist die Blattspreite isoflavonreicher als der Blattstiel. In fast allen Entwicklungsstadien bildet Biochanin A in der Blattspreite den Hauptbestandteil der Isoflavone, während es im Blattstiel das Formononetin ist.

In der Blattspreite bleibt der Formononetingehalt gleich oder fällt mäßig ab. Demgegenüber erreicht der Biochanin A-Gehalt in den ausdifferenzierten Blättern ein Maximum. Im Stiel sind merkliche Unterschiede nicht festzustellen.

Bei einem Vergleich des Isoflavongehaltes in Blattspreite und Blattstiel in Abb. 2 mit dem der gesamten oberirdischen Pflanzenmasse in Abb. 1, ergibt sich eine scheinbare Diskrepanz.

Während der Biochanin-A-Gehalt bei der Ausdifferenzierung der Blattspreite stark ansteigt, sinkt dieser in der gesamten oberirdischen Pflanzenmasse im Laufe der Entwicklung. Es muß in Betracht gezogen werden, daß im Laufe der Entwicklung der Pflanze die Größen- und Gewichtsverhältnisse von Spreite zu Stiel sich wesentlich verändern. Während der Blattstiel beim jungen Blatt 25 % des Blatt-Trockengewichts ausmacht, steigt dieser beim ausdifferenzierten Blatt auf 50—60 %. Dazu kommen noch beim Eintritt in die Blühphase der Blütensproß mit nur kleinen Blättern. Die Blattstiele wie die blühenden Sprosse sind arm an Biochanin A. Mit ihrer anteilmäßigen Zunahme im Laufe der Entwicklung der Pflanze nimmt der Biochanin-A-Gehalt nicht zu, sondern ab.

Es muß erwähnt werden, daß im Rotklee in kleinen Konzentrationen noch weitere Isoflavone vorkommen. So ist Genistein und Daidzein in fast allen Fällen in kleinen Mengen vorhanden. In vorliegender Arbeit wurden diese nicht quantitativ erfaßt.

Wong 1963 berichtete über das Vorkommen von Pratensein in Rotklee, welches sich diazotierter Sulfanilsäure leicht nachweisen läßt. Wegen der geringen Konzentration und geringen oestrogenen Wirkung wurde dem aber nicht gesondert nachgegangen.

Das oestrogen viel wirksamere Cumöstrol wurde versucht nach Livingston, Bickoff, Guggolz und Thompson 1960 und 1961 a sowie nach Grisebach 1963 in Rotklee nachzuweisen. Zur Verbesserung der dünnschichtchromatographischen Trennung wurde eine spezielle Keiltechnik angewendet (siehe Randerath 1962). Cumöstrol konnte aber nicht nachgewiesen werden. Auch Wong 1963 fand in den untersuchten Rotkleearten kein Cumöstrol; ebenso Beck 1964 nicht bei Trifolium subterraneum.

Besprechung der Ergebnisse

Bei dem im Rotklee in relativ hohen Konzentrationen vorkommenden Isoflavonen zeigt Biochanin A, weniger Formononetin eine Anreicherung in der Blattspreite, deren Ausmaß vom entwicklungsmäßigen Zustand des Blattes abhängt. Damit ist es nicht gleichgültig, ob deren Gehalt auf die Gesamtpflanze oder vornehmlich auf einzelne Teile wie die Blattspreite bezogen wird. Dies kann zur Erklärung der Unterschiede dienen, die zwischen den hier vorgelegten Werten an Gesamtpflanzen und den 3—4fach höheren Werten von Blättern nach Wong 1963 bestehen.

Gegenüber den im tierischen Organismus vorkommenden oestrogenen Hormonen bzw. dem häufig als Bezugsbasis verwendeten hochwirksamen Stilboestrol ist die oestrogene Wirksamkeit der ausschließlich in Pflanzen vorkommenden Isoflavone gering. So hat Biochanin A im Vergleich zu Stilboestrol nach Wong und Flux 1962 nur eine $9{,}1\times10^{-6}$fache, nach Nilsson 1961 a eine $7{,}9\times10^{-6}$fache Wirksamkeit. Wong 1963 bezieht sich danach auf einen Mittelwert von $8{,}5\times10^{-6}$.

Bei der Beurteilung der oestrogenen Wirkung der im Rotklee bzw. in anderen Futterleguminosen vorkommenden Isoflavone müssen neben den nachgewiesenen freien Isoflavonen auch die in gebundener Form vorliegenden Isoflavone berücksichtigt werden. Da die enzymatische Freisetzung der gebundenen Isoflavone sehr schnell bei der Zerstörung der Zellen erfolgt, kann angenommen werden, daß dieser Vorgang bereits bei der Futteraufnahme durch das Tier in Gang gesetzt wird. Der vollständige Aufschluß der Pflanzenzellen dürfte allerdings in der Hauptsache mikrobiell im Pansen erfolgen.

Nach oraler Verabreichung von Isoflavonen bzw. isoflavonhaltigen Extrakten an juvenile weibliche Mäuse konnte festgestellt werden, daß die verschiedenen Isoflavonderivate eine unterschiedliche oestrogene Wirksamkeit besitzen. So geben Wong und Flux 1962 das Verhältnis der Wirksamkeit von Genistein : Biochanin A : Daidzein ≈ 1,5 : 1 : 0 : 0,4 an. Nach diesen Autoren war Formononetin im Test unwirksam. Das im Rotklee in niederer Konzentration vorkommende Pratensein hat eine geringere Wirkung als Biochanin A. In weiteren Arbeiten geben Flux, Wilson und Wong 1964 für Genistein und Biochanin A ein Verhältnis von 1,77:1,0 an und reine Substanz sogar von 2,17 : 1,0. Unabhängig davon sind Micheli, Booth, Livingston und Bickoff 1962 bezüglich Genistein und Biochanin A zu ähnlichen Ergebnissen gekommen. Dagegen fanden sie bei den 7-Hydroxy-Isoflavonen Daidzein und Formononetin eine höhere Wirksamkeit als Wong und Flux 1962, die der von den 5,7-Dihydroxy-Isoflavonen Genistein und Biochanin A ähnlich ist. Den Befunden beider Arbeitskreise gemeinsam ist jedoch, daß die oestrogene Wirksamkeit der Isoflavone nach Demethylierung zunimmt.

Daraus ergeben sich Folgerungen für die nach Nilsson 1961 b und 1963 beobachtete Demethylierung von Isoflavonen im Wiederkäuermagen. Danach werden die methylierten Derivate Biochanin A und Formononetin durch fermentative Tätigkeit der Pansenflora in die stärker wirksamen demethylierten Derivate Genistein und Daidzein überführt. Über das Ausmaß dieser Umsetzungen liegen jedoch nähere Angaben nicht vor.

Wird die oestrogene Wirksamkeit der in Rotklee vorkommenden Isoflavone in Stilboestrol-Einheiten berechnet, so muß der Demethylierung im Pansen und damit der erheblichen Wirksamkeitssteigerung der Isoflavone Rechnung getragen werden. Das kommt in der Abb. 3 in der Gegenüberstellung der Wirksamkeit der Isoflavone nach mikrobieller Demethylierung im Pansen (obere Kurve) bzw. der ohne irgendeine Umsetzung, wie diese im Rotklee vorkommen (untere Kurve), zum Ausdruck. Bei vollständiger Demethylierung ergibt sich eine Steigerung der Wirksamkeit auf etwa das Doppelte. Für eine merkliche oestrogene Wirkung sind jedoch diese Isoflavonmengen — auch bei ausschließlicher Fütterung mit Rotklee — nach den gegenwärtigen Erkenntnissen zu gering. Auch Wong 1963 gibt eine 2—7fach geringere Wirkung gegenüber Trifolium subterraneum an. Einzig ein stimulierender Effekt ist zu erwarten.

Unter bestimmten Bedingungen können pflanzliche Oestrogene in kritischen Konzentrationen vorkommen. So ist Voisin 1961 der Ansicht, daß der Oestrogenspiegel im Falle eines Phosphatmangels ansteigt. Ebenso ist die Frage nach weiteren wirksameren Verbindungen z. B. Cumöstrol — das von Guggolz, Living-

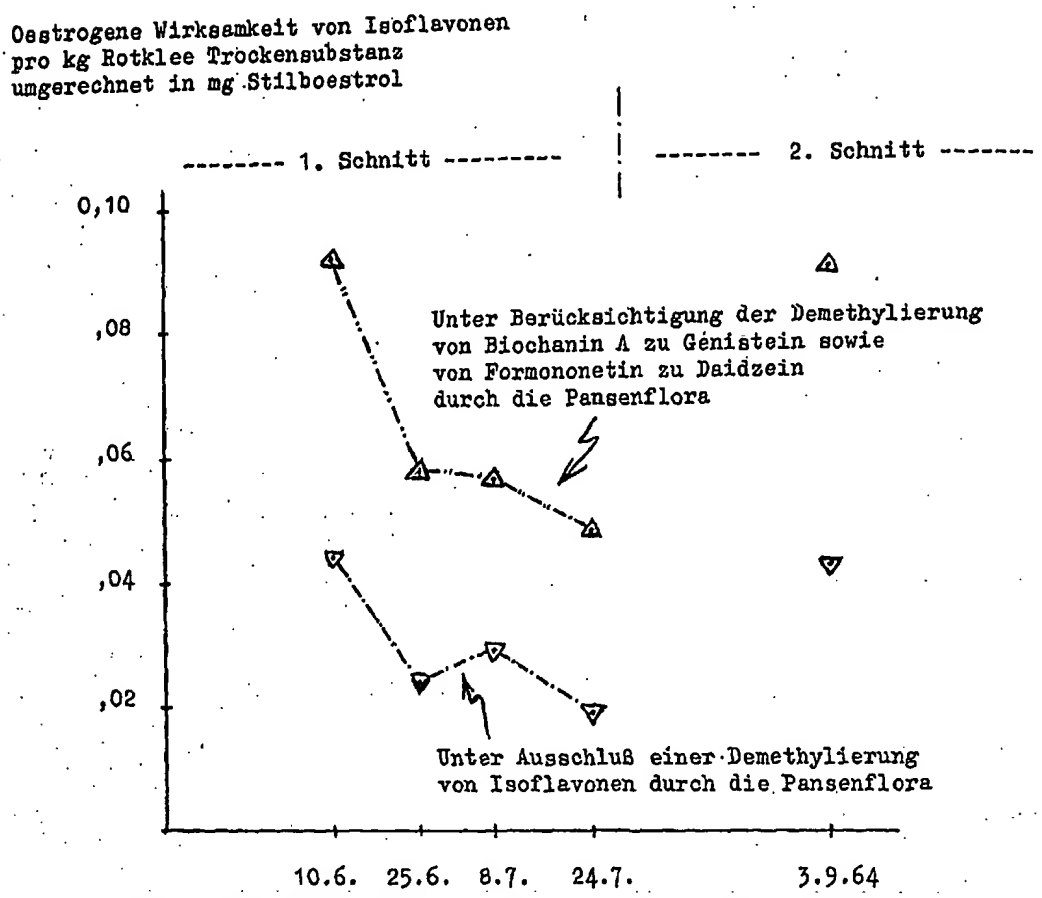


Abb. 3: Oestrogene Wirksamkeit der Isoflavone aus Rotklee entsprechend mg Stilboestrol (berechnet aus den Werten der Abb. 1.

Obere Kurve: Bei Berücksichtigung der Demethylierung von Biochanin A bzw. Formononetin durch die Pansenflora — Untere Kurve: Unter Ausschluß einer Demethylierung von Isoflavone. — Umrechnungsfaktoren auf die oestrogene Wirksamkeit von Stilboestrol: Obere Kurve: Biochanin A (aus Abb. 1) — wegen Demethylierung zu Genistein — mit dem Faktor $1{,}77 \times 8{,}5 \times 10^{-6}$ multipliziert; Formononetin — wegen Demethylierung zu Daidzein — mit dem Faktor für Daidzein $0{,}4 \times 8{,}5 \times 10^{-6}$ multipliziert.

ston und Bickoff 1961 auch im Rotklee nachgewiesen werden konnte, noch nicht abgeklärt.

Zusammenfassung

1. Der Gehalt an Formononetin (7-Hydroxy-4'-methoxy-isoflavon) (Fo) und Biochanin A (5,7-Dihydroxy-4'-methoxy-isoflavon) (BA) wurde in einem Freilandversuch mit T. pratense untersucht.

a) Bezogen auf die Trockenmasse von Sproß plus Blatt bleibt der Gehalt an Fo im vegetativen Stadium der Pflanze annähernd konstant (etwa 0,4 %). Der Gehalt an BA sinkt dagegen im Laufe der Ausdifferenzierung der Pflanze auf etwa die Hälfte.

b) Werden Blattspreite und Blattstiel getrennt untersucht, so zeigt der Blattstiel einen sehr geringen Gehalt an BA, während dieser in der Blattspreite i. a. höher (>1 %) als der Gehalt an Fo ist. Die Abnahme des Gehalts an BA in der Gesamtpflanze ist auf die anteilmäßige Zunahme der Blattstielmasse im Laufe des Wachstums zurückzuführen.

c) Die beiden Isoflavone sind erst nach Hydrolyse der Trockensubstanz vollständig als Aglukone erfaßbar.

2. Die oestrogene Wirkung der in T. pratense vorkommenden Isoflavone wird diskutiert.

Schultz, G.: Oestrogen-effective isoflavones in Trifolium pratense (red clover). Distribution in subterranean parts of plants and occurrence as „bound" isoflavones

Summary

1. The content of formononetin (7-hydroxy-4'-methoxy-isoflavon) (Fo) and biochanin A (5,7-dihydroxy-4'-methoxy-isoflavon) (BA) was tested in a planting experiment with T. pratense.

a) In relation to the dry mass of sprout and leaf, the content of Fo remains approoximately constant (≈ 0.4 %) in the vegetative stage of the plant. The content of BA, however, decreases in the course of differentiation of the plant to approximately the half.

b) If the leaf and stalk are tested seperately, the stalk has a very low content of BA, whereas in the leaf the latter is higher (> 1 %) than that of Fo. The decrease of BA in the total plant is due to the increase of the stalk mass in the course of growth.

c) Both isoflavones can be demonstrated as aglucones only after hydrolysis of the dry substance.

2. The oestrogenic effect of the isoflavones occuring in T. pratense is discussed.

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Nonsteroidal estrogens of dietary origin: possible roles in hormone-dependent disease[1,2]

KDR Setchell, SP Borriello, P Hulme, DN Kirk, and M Axelson

ABSTRACT Equol, a nonsteroidal estrogen of dietary origin, was recently identified in human urine, and is excreted in amounts comparable to the classical steroidal estrogens. We confirm here that phytoestrogens which are abundant in dietary soya protein are converted by human gastrointestinal flora to this weak estrogen. After the ingestion of meals containing cooked soya protein the urinary excretion of equol in four of six subjects studied increased by up to 1000-fold and this compound was the major phenolic compound found in the urine. These data also indicate that some subjects are unable to either produce or excrete equol despite the challenge of a diet containing soya. In view of the increasing use of commercial soya products in the diet and the capacity of human bacterial flora to synthesize this weak estrogen from the abundance of phytoestrogens in soya, the potential relevance of these observations to the diseases implicating steroid hormones is discussed. *Am J Clin Nutr* 1984;40:569–578

KEY WORDS Soyabean, dietary estrogens, phytoestrogens, gut bacterial metabolism, urinary equol, menstrual cycle disorders, breast cancer

Introduction

Our recognition of the presence of relatively large quantities of a variety of phenolic compounds in biological fluids (1–3) has aroused interest in the potential biological and physiological importance of this type of compound in man (4).

Several of these phenolic compounds, which are excreted in quantitites much greater than the classical estrogenic (phenolic) steroids, were recently identified as belonging to the chemical class called lignans (2, 3, 5). As a consequence of our studies on lignans it was apparent that in addition to estrogens, several other phenolic compounds were present in biological fluids, and structural elucidation studies revealed the identity of one of these to be equol, [7-hydroxy-(4′-hydroxyphenyl)chroman] (6).

Equol, a phytoestrogen that is structurally similar to estradiol-17β, (Fig 1) was first discovered in the urine of mares (7) and later found in urine from goats (8), cows (9), hens (10, 11), and sheep (12, 13) but had never previously been identified in man. Of particular significance, is the fact that this phytoestrogen possesses weak estrogenic activity (12–14), while also behaving as an antiestrogen in exhibiting a competitive binding with estradiol-17β for uterine cytosol receptors (14, 15). It was subsquently shown to be the "contraceptive" agent responsible for an infertility syndrome, referred to as "Clover disease," which was widespread in Australian agricultural animals (16–18).

Equol is not present in plants in significant quantities, but other phytoestrogens related in structure, such as formononetin, daidzein, and genistein (Fig 1) are found extensively throughout the plant world (19). In animals, equol is formed in the gastrointestinal tract as the result of the bacterial degradation of these phytoestrogens which are ingested in relatively large quantities in the feed (13, 20–24), and data presented here

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confirm that equol may be formed similarly in man.

From previous observations, soya was found to be a foodstuff rich in precursors which could be readily converted into the weak estrogen equol (6, 24, 25). In view of the increasing use of soyabean as a protein food source in the Western world, and the general acceptance of diet as a major factor in disease, the role of biologically active phenolic compounds, such as phytoestrogens (26, 27) and lignans (2–5), which are present in our diet (25, 28), requires future consideration. The potential relevance of exposure to phytoestrogens in patients with menstrual cycle irregularities, infertility, and breast cancer, and in infants is discussed.

Experimental

Subjects

Six healthy laboratory personnel, four men and two women (age range 22 to 39 yr) were studied. Urine samples (24 h) were collected over a 14-day period, the volumes recorded, and aliquots taken and stored at −20°C. After the first 3 days of "normal" unrestricted diet, soya was substituted at one main meal for a period of 5 consecutive days. Textured soya (Natural Protoveg) was obtained from Direct Foods Ltd, Petersfield, Hants, UK and its chemical composition consisted of 52% protein, 3.5% carbohydrate, and 1% fat. Each subject received 40 g dry weight per day which was cooked according to the manufacturers instructions. On day 9 of the study the diet reverted to normal.

In vitro incubations with fecal flora

One g of freshly voided feces was added to each of a 9-ml volume of sterile distilled water, trypticase soy broth (BBL) with the addition of 1 g of soy protein, and brain-heart infusion broth (Difco). Uninoculated broths served as negative controls. All broths were incubated anaerobically for 3 days at 37°C. Postincubation the sample was treated as described below.

Determination of equol

The technique for the determination of equol in urine samples and fecal incubations is described in detail elsewhere (6, 24, 25) and is briefly outlined.

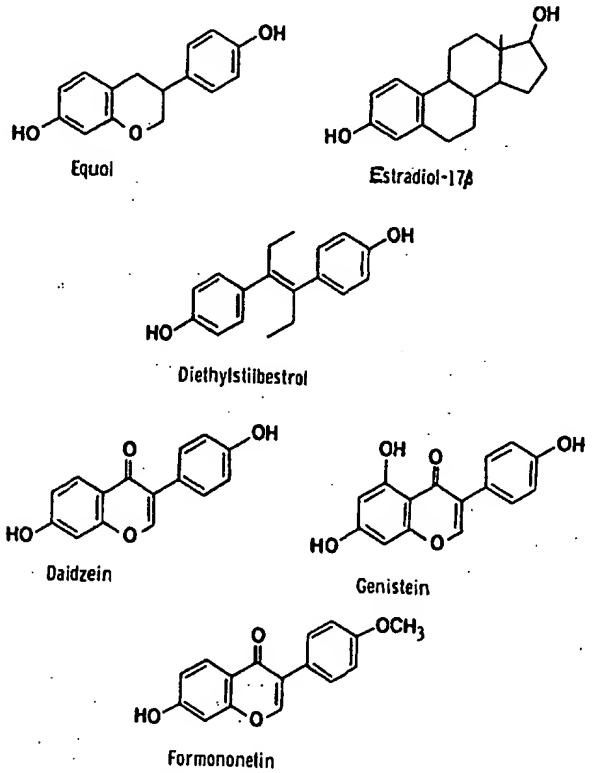

FIG 1. A comparison of the chemical structures of *1*) the phytoestrogen equol, formed in the gastrointestinal tract of man and animals, *2*) estradiol-17β, *3*) the potent synthetic oestrogen, diethylstilbestrol, and *4*) several phytoestrogens of plant origin.

Urine. Equol, which is present in urine as the glucuronide conjugate (6, 24, 25), was extracted together with steroids and lignans using reverse phase octadecylsilane bonded silica cartridges. After hydrolysis of the conjugate by a β-glucuronidase preparation (*Helix pomatia*) and reextraction using the same extraction procedure, equol was isolated and purified by gel chromatography using either a straight phase partition system on Sephadex LH-20 (29) or by ion-exchange on a lipophilic ion exchange gel, TEAP-Sephadex-LH-20 (24). 5α-Androstane-3β,17β-diol was added as an internal standard and the fraction containing equol was converted to the trimethylsilyl ether derivative. Quantification of equol was performed by gas chromatography (24) with flame ionization detection or by selected ion monitoring gas chromatography mass spectrometry using the ions m/z 386 and 346, which are characteristic ions in the mass spectra of the trimethylsilyl ether derivatives of equol (6) and 5α-androstane-3β,17β-diol, respectively. Authentic samples of equol were obtained from the MRC Steroid Reference Collection, Westfield College, London and from Prof H Adlercreutz (Department of Clinical Chemistry, Meilahti Hospital, University of Helsinki, Finland).

Fecal cultures. The incubation sample (2 ml) was diluted with an equal volume of distilled water and centrifuged at 3500 rpm for 5 min. The supernatant was passed through a cartridge of reverse phase octadecylsilane bonded silica to extract equol. Equol was recovered from the cartridge with methanol, isolated by gel chromatography, and analyzed by gas chromatography as the trimethylsilyl ether derivative after addition of the internal standard as described above.

Results

The gas chromatographic analysis of the urine from one of the subjects (JG) before and 3 days after the ingestion of soya protein, is compared in Figure 2. Before soya ingestion, equol was not detectable in urine (<5 μg/day), whereas after soya it became the major component of the phenolic fraction. Its excretion in the urine from this subject was 5.3 mg/day at this time, which is considerably greater than the two recently discovered phenolic lignans, enterolactone and enterodiol (1–5), and vastly in excess of any of the urinary estrogens, which could not be detected by gas chromatography.

The urinary excretion of equol in four of the six healthy adults studied, before, during, and after the ingestion of cooked soya protein (40 g/day) is shown in Figure 3. Basal levels of equol ranged from undetectable (<5 μg/day) to a maximum of 80 μg/day. After the consumption of soya, four of the six subjects (two men and two women) showed a marked increase in the urinary excretion of equol within 1 day, with the excretion increasing by 50- to 1000-fold. The maximum urinary excretion of equol ranged from 3.5 to 7.0 mg/day during the period of soya ingestion. After the diet reverted to normal and in the absence of any known source of soya, the urinary excretion of equol gradually returned to <100 μg/day within several days. In two of the subjects, both men, equol was not detected in the urine and a challenge of soya appeared to have no effect on equol excretion in one of these subjects and a negligible effect in the other (subject MM, Fig 3).

In vitro incubation of soya protein with the fecal flora obtained from one of these subjects (JG) confirmed their ability to form equol. Equol was only found in the soya broth after incubation with fecal bacteria. A comparison of the gas chromatographic analysis of the phenolic compounds isolated from a sample of (*a*) stool + diluent + soya and (*b*) stool + diluent is shown in Figure 4. For reference (*c*) authentic equol and the internal standard, 5α-androstane-3β,17β-diol are shown. Equol was not detectable in the sample of stool analyzed but was the major component after coincubation of the stool with a soya rich broth.

Discussion

Our earlier studies have shown that in addition to estrogens in biological fluids, there are many other phenolic compounds (1–4, 6) and that these are excreted in much higher concentrations than the classical steroidal estrogens (30, 31).

We recently reported the identity of two novel diphenolic compounds (2), enterolactone [2,3-bis(3-hydroxybenzyl)butyrolactone] and enterodiol [2,3-bis(3-hydroxybenzyl)butane-1,4-diol], which are chemically classified as lignans, and after these studies an isoflavan, equol, which is also diphenolic in structure was identified (6). Equol is classified as a phytoestrogen and possesses weak estrogenic activity, of the order of 10^{-3} of that of estradiol-17β (14, 15). Structurally these compounds are similar to estradiol-17β (Fig 1) and the potent estrogen, diethylstilbesterol (DES) in possessing a phenyl substituent which is considered as one of the

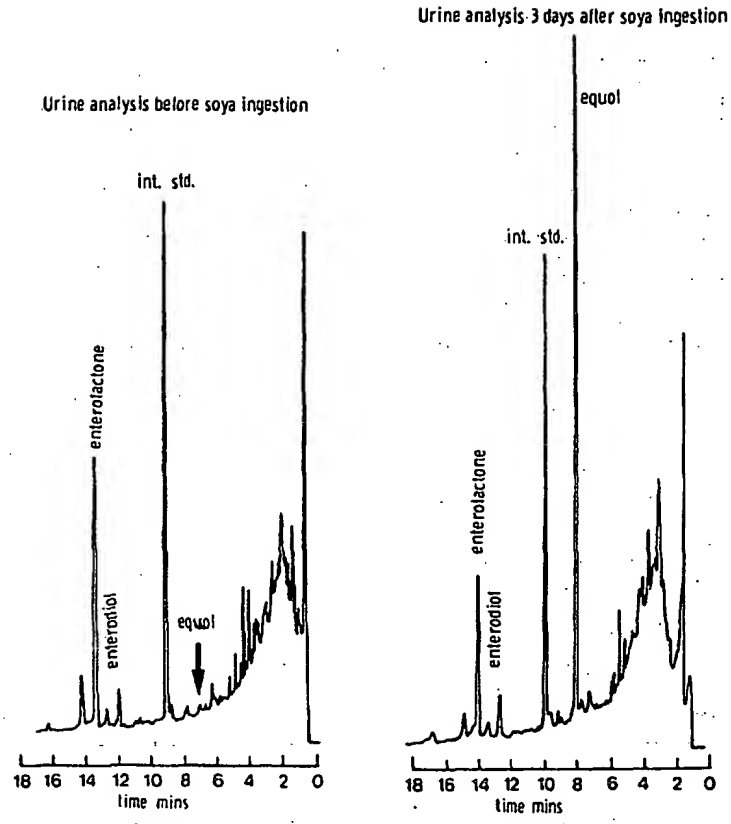

FIG 2. A comparison of the gas chromatograms of the trimethylsilyl ether derivatives of phenolic compounds isolated from the urine of an adult before and after the ingestion of 40 g of cooked soya protein. Gas chromatography was carried out on a 25 m chemically bonded silicone SE-30 column using isothermal operation at 250°C. 5α-Androstane-3β,17β-diol was the internal standard added and the identity of equol was confirmed by gas chromatography–mass spectrometry by comparison with the authentic compound.

prerequisites for estrogenic activity (32). In the last decade considerable attention has focussed on the potential deleterious effects in humans of the use of DES as a growth promoter in animal feed (33, 35). This follows the adverse effects reported after the therapeutic use of the drug for prostatic cancer (36–38) and of its carcinogenicity when administered in low doses in animals (39–41).

Although there are no reports of the effects in humans of DES in food, until its withdrawal from use in the Western world, animal tissue was strictly monitored at levels as low as 1 ppb. The level of naturally occurring nonsteroidal estrogens in many foods is substantially higher than the concentration of DES in animal tissues and the implications of this, as far as human disease is concerned, requires careful consideration, particularly when it is becoming increasingly accepted that many diseases common to the Western world are associated with dietary factors (42).

Our earlier studies using rats (6, 24) revealed that equol was excreted in urine and bile in amounts in excess of the lignans enterolactone and enterodiol. These studies also proved that, in rats, equol was formed by gut bacteria and that it undergoes an enterohepatic circulation, in common with many endogenous compounds, including estrogens (43, 44). In animal experiments designed to determine the source of precursors

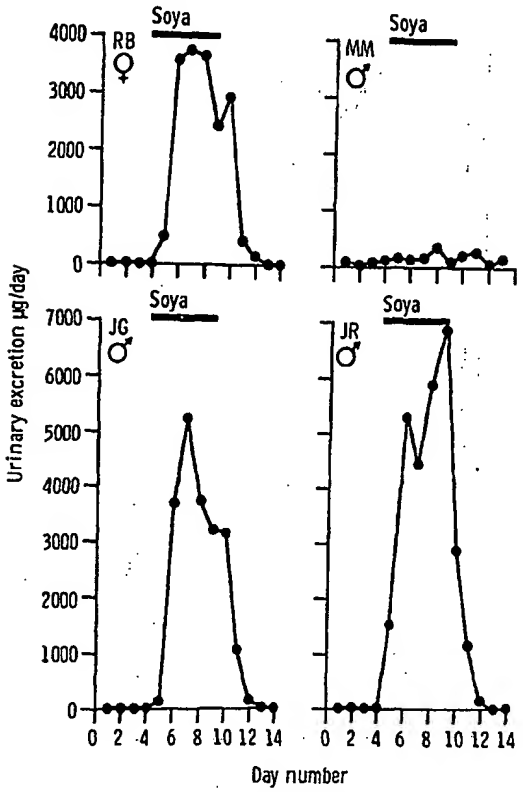

FIG 3. The urinary excretion (µg/day) or equol in four subjects over a 14-day period in which cooked soya protein (40 g) was ingested for 5 consecutive days.

to these compounds (25, 28) it was found that when soyabean was fed to rats a marked increase in the urinary excretion of equol occurred (6, 25). Data presented here confirm that soya is rich in precursors which are also used by human gut flora to give rise to substantial quantitites of equol. In vitro studies establish the ability of human fecal microflora to perform the necessary reactions to degrade phytoestrogen precursors to equol, however, in vivo it was found that only four of the six subjects studied excreted equol in the urine. The reason for this is unknown, and in view of the limited number of subjects studied, it is impossible to determine what proportion of the population are "nonresponders." Since both males and females are capable of excreting large quantities of equol after soya ingestion it would appear to be sex independent. The rate of formation of equol from daidzein, the phytoestrogen precursor in soya (25), is presumably influenced by the composition of intestinal microflora, the intestinal transit time, and alterations in the redox level in the large intestine, factors that may be strongly influenced by diet.

The urinary excretion of equol in humans consuming diets with no obvious major source of soya protein is less than 80 µg/day and this is in accord with levels reported recently (6, 45). After the consumption of a single meal consisting of 40 g of soya each day for 5 consecutive days the peak urinary excretion of equol exceeded 3.5 mg/day, representing in some cases an increase of up to 1000-fold in this weak estrogen. Compared with levels of the principal urinary estrogen, estrone-glucuronide, which in the follicular phase of women is excreted in

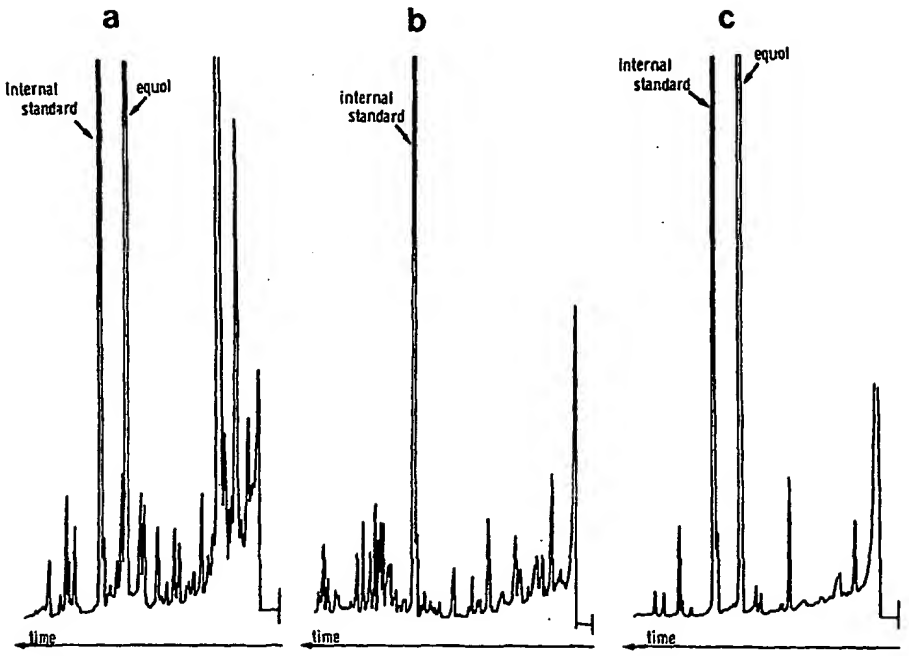

FIG 4. Gas chromatograms of the trimethylsilyl ether derivatives of phenolic compounds isolated from (*a*) human fecal flora, diluent, and soya protein, and (*b*) human fecal flora and diluent after a 3-day incubation period. For comparison, the trimethylsilyl ethers of an authentic sample of equol and the internal standard are shown in chromatogram (*c*). Gas chromatography was performed using a 25 m chemically bonded silicone SE-30 column and temperature programmed conditions from 185 to 275°C in increments of 2°C/min.

amounts ranging from 2 to 27 μg/day (29, 30), equol excretion after the ingestion of soya protein is significant and raises the question of the physiological relevance of a large amount of this weak estrogen.

The presence of phytoestrogens in soyabeans has been recognized for some time (46–48) and its estrogenicity has been reported (49) after observing that cake containing soya produced uterotrophic effects in rats (50). Daidzein and genistein (Fig 1) have both been reported to occur in soyabean in substantial amounts (46–48) [although we have been unable to confirm the presence of the latter phytoestrogen in the preparations of soya used in these studies (25)], and it was suggested that because of the estrogenicity of these compounds, soya cake might be as beneficial as diethylstilbestrol as a growth promoter in agricultural animals (48).

Our earlier finding of large amounts of equol in the urine of rats (24) and our recent identification of daidzein as the major phytoestrogen in soya flour (25) indicate that daidzein is converted by gut microflora into the more potent estrogen equol. The effects reported earlier in rats were therefore probably induced by equol rather than genistein and/or daidzein as was originally suggested (49).

In view of the various reproductive disorders in animals that have been associated with the ingestion of a variety of phytoestrogens (16, 18, 49–52), consideration should be given to the possible effects in man of the large quantities of equol that are derived from the ingestion of soyabean products. There may be some value therefore, in assessing the dietary status and determining the levels of phytoestrogens in biological fluids of patients with menstrual cycle disorders and in cases of infertility where there are no obvious physiological abnormalities. The potential value of plants as sources of antifertility agents (19, 53) gained impetus

after reports of the "contraceptive" action of equol in Australian sheep grazing on *Trifolium subterraneum*, a species of clover containing large quantities of the phytoestrogen, formononetin (16) (Fig 1). Furthermore a reduction in sperm count has been reported in sheep grazing for prolonged periods on this clover (54). In view of these effects in animals, it is conceivable that similar actions may occur in humans consuming diets rich in phytoestrogen precursors, provided that the intestinal flora responsible for their conversion to equol are both present and active.

Breast cancer, one of the most common causes of death from cancer in women, has long been known to be associated with hormone activity (55), but diet is also suspected to be a major factor in its etiology (42, 56, 57). Animal models, such as the 7,12-dimethyl-bene(α)anthracene-induced rat mammary carcinoma which is estrogen dependent (58), have helped in the understanding of the hormone dependence of mammary tumors, and estrogen receptors similar to those found in the uterus and vagina have been found in human breast cancer tissue (59, 60). Although the mechanism of action of estrogens on tumor growth is not fully understood, estrogen receptors are sensitive to both estrogens and antiestrogens (32). Estrogens bind to the appropriate cytoplasmic receptor, eliciting its translocation to the nucleus with subsequent retention of the estrogen-receptor complex. These events lead to an increase in RNA synthesis, which in turn results in protein synthesis and cell growth. Conversely, antiestrogens are considered to exert their effect by decreasing the cytoplasmic estrogen-receptor concentration (61), thereby producing an insensitivity of the target tissue to estrogen stimulation (62) and by forming complexes with the receptor preventing the initiation of biosynthetic events leading to tissue growth.

Several phytoestrogens have been demonstrated to inhibit the binding of estradiol-17β to uterine cytosol receptors (14, 15, 26) indicating that structurally a steroid nucleus is not essential for binding to the estrogen receptor. Structurally, the distance between the C-3 and C-17 hydroxyl groups of estradiol-17β is similar to that of the two hydroxyl groups of the aromatic rings of phytoestrogens (compare structures in Fig 1), a factor that is esential for strong binding to the estrogen receptor. The effect of equol in binding to the receptor in the nucleus but failing to stimulate DNA synthesis to the same degree as estradiol-17β, is supportive of an antiestrogenic role for this phytoestrogen (14).

As far as mammary tissue is concerned, several phytoestrogens have been shown to bind to estrogen receptors of human breast cancer cells (63). However, to our knowledge equol has not been tested but the plant phytoestrogens, coumesterol, commonly found in legumes and zearalanol, a mycotoxin that contaminates grains, have been shown to bind competitively to the estrogen cytosol receptor of both rat and human mammary tumour tissue (27, 63). Estrogens exert dose dependent dual effects upon tumour induction and growth. High doses inhibit tumour development and suppress growth (64, 65) while physiological doses stimulate growth of human tumour cells (66, 67). The significance therefore of naturally occurring phytoestrogens, which may also exhibit a similar dual role and which are consumed in our diet and synthesised in the gut, to the etiology of human breast cancer or its therapy is not known. In view of the affinity of these diphenolic compounds for estrogen receptors, the effects of exposure to high levels of a compound such as equol, which as we have demonstrated can occur after the ingestion of soyabean products, require examination. So while we hypothesize that repeated soya consumption in man may result in reproductive disorders due to the estrogenic effects of equol or other phytoestrogens, similar to its action in animals, conversely, its antiestrogenic effects may be beneficial with respect to breast cancer development or in its treatment.

Finally, with the recent introduction of soya milk products for infant feeding it would be of interest to determine how the newborn infant handles and metabolizes the phytoestrogens which are present in large quantities in the soya [25, 46–49]. Although the gut is sterile at birth, during the 1st wk of life it rapidly develops a bacterial flora (68). Whether in early life the bacterial enzymes which in adults are responsible for

the conversion of daidzein-glycoside in soya to equol are present, remains to be established. If so, the infant may be subjected to concentrations of this weak estrogen which are well in excess of endogenous estrogen levels, and if not, it is probable that the precursor, daidzein, itself a weak estrogen, may be absorbed and excreted in the urine as the glucuronide conjugate, as shown in adults (25, 69). Therefore, the potential effects of subjecting infants as well as adults to relatively large amounts of dietary phytoestrogens remains to be evaluated.

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Isoflavone in einigen Weiß- und Rotkleesorten und ihre oestrogene Wirksamkeit bei juvenilen Mäusen

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Oestrogenhaltige Stoffe in Pflanzen wurden bereits von Dohrn und Mitarbeitern 1926 gefunden. Erste Beobachtungen über pathologische Oestrogenwirkungen machten Bennets und Mitarbeiter (1946) in Australien bei Schafen nach Verfütterung von Trifolium subterraneum. Nymphomane Erscheinungen sowie übermäßige Euterentwicklung und Milchsekretion bei nichttragenden Färsen beobachteten Adler und Trainin (1953) nach Verabreichung von Luzerne. Auch Pope (1954), Schoop und Klette (1955) sowie Schoop (1957) und Rankin (1963) berichteten über pflanzenoestrogenbedingte Laktation bei juvenilen Färsen. Aborte sowie Zyklusstörungen in Rinderherden nach Aufnahme von oestrogenhaltigen Pflanzen sahen Drejare (1962) und Rankin (1963).

Bradbury und White (1961) zeigten, daß diese bis ins Pathologische gesteigerte Oestrogenaktivität den in einigen Leguminosen enthaltenen Isoflavonen, besonders dem Genistein (5, 7, 4'-Trihydroxyisoflavon) zuzuschreiben ist (Zusammenfassung der älteren Literatur bei Moule, Braden und Lammond [1963]).

Auch der in Mitteleuropa häufig angebaute Rotklee (Trifolium pratense) enthält Isoflavonderivate, die im Vergleich zu Genistein allerdings schwächer wirksam sind, so das Biochanin A (4'-Methyläther des Genisteins) und das sehr wenig wirksame Formononetin (4'-Methyläther des Daidzeins = 7, 4'-Dihydroxyisoflavon) (Wong [1962, 1963]). Biochanin A ist $8{,}5\times10^{-6}$mal weniger wirksam als Diaethylstilboestrol (Wong [1963]). Die Wirkung stuft sich vom Genistein über Biochanin A zu Daidzein im Verhältnis 1,5:1:0,4 ab (Wong [1963]). Formononetin ist nach Ansicht dieses Autors inaktiv. Allerdings erfolgt nach Nilsson (1961, 1962) im Pansen durch Demethylierung eine Umwandlung von Biochanin A in Genistein sowie von Formononetin zu Daidzein und damit eine Wirkungssteigerung. Wie Untersuchungen an Ratten ergaben, findet die Umwandlung ebenfalls in der Leber statt (Nilsson [1961]).

Der Oestrogengehalt der verschiedenen Weißkleesorten ist nach Angaben der Literatur unterschiedlich. Die in den USA von Bickoff und Mitarbeitern (1957, 1958) untersuchte, sehr frohwüchsige Varietät Ladino (Trifolium repens var. typicum f. Lodigense, „Ladino" [siehe Hegi]) enthält das stark oestrogenwirksame Cumoestrol, das biogenetisch mit Formononetin verwandt ist (Grisebach und Barz [1963, 1964]). Im Gegensatz zu Formononetin stellt Cumoestrol ein relativ wirksames Pflanzenoestrogen dar und ist etwa 30- bis 40fach stärker als Genistein (siehe Schauer [1963]).

Von den in Europa gebauten Weißkleesorten wurde bisher nur die dänische Varietät Morsoe Ötofte untersucht, bei der im Tierversuch keine oestrogene Wirkung nachgewiesen werden konnte (Kallela [1964]).

Versuchsanordnung

Die Kleesorten wurden im Frühjahr 1966 auf sandig-humosem Boden, der 1965 mineralisch gedüngt worden war, ausgesät. Wegen geringer Sorptionskraft des Bodens und der Möglichkeit des Stickstoff- und Kalimangels wurde im Mai 1966 mit Kalisalpeter (10 g N/m^2) gedüngt. Die Ernte der Blattspreiten (Blattlamina) erfolgte Anfang Juli. Die Pflanzen zeigten zu diesem Zeitpunkt noch keine Anzeichen der Blütenbildung. Das Blattmaterial wurde bis zur Verarbeitung tiefgefroren. Die Extraktion erfolgte unter Berücksichtigung der Erfahrungen von Bickoff und Mitarbeitern (1959): 500 g Frischsubstanz wurden 16 Std. im Soxhlet mit 3000 ml Methanol (chemisch rein) extrahiert und anschließend das Methanol abdestilliert. Den verbleibenden Rest nahmen wir mit 50 ml Aqua dest. auf und versetzten ihn mit 150 ml Aqua dest. im Scheidetrichter. Danach erfolgte die Ausschüttelung der in der wäßrigen Phase befindlichen wenig polaren Lipide, Chlorophylle, Carotinoide u. ä. mit 8×250 ml Petroläther Kp. 60—70°. Der so gereinigte Extrakt wurde auf ein kleines Volumen in vacuo eingeengt und mit 200 ml Aqua dest. aufgenommen. Die Isoflavone wurden mit 6×100 ml Äther ausgeschüttelt, der Äther abdestilliert und der Rückstand anschließend in 15 ml Äther DAB 6 gelöst. Diese Lösung wurde mit Mäusefutter (Pellets) vermengt, der Äther im Trockenschrank abgedampft. Das Futter der Kontrolltiere behandelten wir mit Äther ohne Extraktstoffe. Bei der Extraktion muß ein Reinigungsverlust von etwa 50 % in Betracht gezogen werden.

Je Versuchsgruppe standen 5 juvenile Mäuse (Gewicht 8—10 g) zur Verfügung. Die Fütterungsdauer betrug 5 Tage. Jede Maus erhielt pro Tag 2 g des Fertigfutters, präpariert mit dem Extrakt von 20 g Klee (Frischgewicht).

Bei den Mäusen wurde der Zeitpunkt der Öffnung des Scheideneinganges und des Vaginalhäutchens (Vaginalöffnungstest) und der Verhornung des Scheidenepithels (Allen-Doisy-Test) registriert. Am 6. Versuchstag wurden Kontroll- und Versuchstiere getötet und die Bestimmung des Uterusgewichtes vorgenom-

Übersicht 1: Oestrogenwirkung nach Verabreichung von Klee-Extrakten im Futter (Extraktmenge pro Tier entsprechend 100 g Kleefrischgewicht) sowie Isoflavongehalt des Methanolextraktes aus Klee.

	Zahl der Versuchstiere	Vaginalöffnung Versuchstag 1.	2.	3.	4.	5.	Verhornung des Scheidenepithels Versuchstag 1.	2.	3.	4.	5.	Uterusgewicht mg/100 g Körpergewicht[1])	Isoflavongehalt im Methanolextrakt in mg/kg Klee (Blattspreite) Biochanin A	Formononetin	Daidzein
Kontrolle	5	—	—	—	—	—	—	—	—	—	—	66,2 ± 6,2			
Weißklee:															
v. Kameke	5	—	—	+	+	+	—	—	—	—	—	66,8 ± 7,1	—	19,6	14,0
NFG Gigant	5	—	—	—	—	—	—	—	—	—	—	66,0 ± 3,3	—	26,7	14,0
dän. Morsoe	4	—	—	—	+	+	—	—	—	—	—	68,0 ± 3,9[2])	—	26,6	8,4
Rotklee:															
Lembke	5	—	—	+	+	+	—	—	—	+	+	112,1 ± 16,4	392	560	—
Ostsaat Treu	5	—	—	+	+	+	—	—	—	+	+	127,3 ± 21,2	448	476	—
niederrh. Remy	5	—	—	+	+	+	—	—	—	—	—	64,0 ± 4,5	532	672	—

[1]) Standardabweichung des Mittelwertes $s_{\bar{x}}$.
[2]) Unter Nichtberücksichtigung eines Tieres mit Eigen-Oestrogenaktivität (stark pos. Vaginalverhornungs- und Scheidenöffnungstest am 1., aber nicht mehr am letzten Versuchstag).

men. Um bessere Vergleichsmöglichkeiten zu haben, erfolgte die Umrechnung der Uterusgewichte auf 100 g Körpergewicht nach Bülbring (1935). Parallel dazu wurden analytisch die einzelnen Isoflavone nach dünnschichtchromatographischer Trennung bestimmt (Senultz [1965]).

Ergebnisse und Diskussion

In Tabelle 1 sind das nach 5tägiger Versuchsdauer ermittelte Gewicht der Uteri, der Zeitpunkt der Öffnung der Scheide, der Zeitpunkt und Grad der Verhornung des Scheidenepithels sowie der Isoflavongehalt der Kleesorten angegeben.

Den 3 Weißkleesorten v. Kameke, NFG Gigant und dän. Morsoe gemeinsam ist eine so schwache oestrogene Wirksamkeit, daß sich die Uterusgewichte nur um 3 %, d. h. in keiner Weise signifikant von den Kontrollen unterscheiden. Ebenso ist der Vaginalverhornungstest in allen Fällen sowie der Scheidenöffnungstest bei den mit dem Extrakt von T. repens NFG Gigant gefütterten Tieren negativ. Darin liegt der klare Unterschied zu dem von Bickoff und Mitarbeitern (1959) untersuchten Ladino-Klee. Nicht geprüft wurden die Wurzeln auf ihren Cumoestrol-Gehalt. Beispielsweise stellt die Wurzel bei Luzerne (Medicago sativa) die Hauptquelle für die Isolierung von Cumoestrol dar (Grisebach und Mitarbeiter [1963]). Die Versuchsdauer von 6 Tagen erscheint genügend lang, um Veränderungen an den Geschlechtsorganen der Versuchstiere auszulösen, falls in den zugeführten Extrakten Oestrogene vorhanden gewesen wären. So hat Elghamry (1963) nach oraler Verabreichung von Ladino-Extrakten an Ratten schon nach einer Woche etwa eine 3fache Vergrößerung des Uterusgewichtes erreichen können, ein Wert, der sich in der 2. und 3. Woche der Behandlung kaum mehr erhöht und in der 4. Woche sogar unter die Kontrollen absinkt.

Die Rotkleesorten Lembke und Ostsaat-Treu weisen beide im Mäusetest eine Oestrogenwirkung auf, die sich in positivem Vaginalverhornungstest, Öffnung der Scheide innerhalb von 3 Tagen und Vergrößerung des Uterusgewichtes um 70 (bei Lembke) bzw. 90 % (bei Ostsaat-Treu) äußert. Die Biochanin A-Verabreichung von etwa 20 bzw. 22,5 mg pro Tier (eingerechnet den Reinigungsverlust von etwa 50 %) war genügend hoch. Schauer (1964) gibt für Biochanin A als Dosis zur Erhöhung des Uterusgewichtes bei Mäusen auf das 2,5fache etwa 18 mg an. Unsere Werte stimmen damit annähernd überein. Außer Biochanin A wurde in den von uns untersuchten Rotkleesorten auch das Isoflavon Formononetin (392—672 mg/kg Klee) nachgewiesen. Trotz des hohen Formononetin-Gehaltes kam es zu keiner weiteren Erhöhung der Uterusgewichte über das 2,5fache hinaus. Somit kann geschlossen werden, daß Formononetin kaum wirksam ist. Gleiche Schlußfolgerungen zogen bereits Wong und Flux (1962).

Die Rotkleesorte niederrh. Remy weicht insofern ab, als sie in diesem Versuch keine Oestrogenwirksamkeit entfaltet, aber trotzdem Biochanin A in Mengen aufweist, die höher als bei den Sorten Lembke und Ostsaat-Treu liegen. Bevor jedoch die Frage etwaiger Anti-Oestrogene aufgeworfen wird, sollten — sobald entsprechendes Material vorliegt — Nachprüfungen vorgenommen werden.

Zusammenfassung

Aus den Extrakten der Weißklee-Sorten v. Kameke, NFG Gigant und dän. Morsoe konnten dünnschichtchromatographisch nur die Isoflavone Formononetin und in geringer Konzentration Daidzein, jedoch nicht Cumoestrol nachgewiesen werden. Bei der biologischen Nachweisreaktion an juvenilen Mäusen traten keine oestrogenen Wirkungen auf. Im Gegensatz dazu verlief der Tierversuch nach Verabreichung der Extrakte aus den Rotklee-Sorten Lembke und Ostsaat-Treu positiv. In den Rotklee-Extrakten wurden die Isoflavone Biochanin A und Formononetin in größerer Menge gefunden.

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Grunert, E., G. Woelke and G. Schultz: Isoflavones in some white and red clover varieties and their oestrogenic effect in juvenile mice.

Summary

Extracts of the white clover varieties Danish-Morsoe, NFG Gigant and v. Kameke were ineffective in the test with immature mice (vaginal opening, vaginal smear and uterine weight). After thin layer chromotography Coumestrol could not be found, only the isoflavones formononetin and daidzein in low concentration. In contrast red clover varieties Lembke and Ostsaat-Treu are effective; the isoflavones biochanin A and formononetin are present in higher quantities.

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Soya – a dietary source of the non-steroidal oestrogen equol in man and animals

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ABSTRACT

The dietary origin of the weak oestrogen equol (7-hydroxy-3-(4′-hydroxyphenyl)-chroman) present in human urine has been investigated using gas chromatography–mass spectrometry. Feeding experiments with different food constituents and monitoring the urinary excretion of equol revealed that soya food yields more than 0·1 mg urinary equol/g flour ingested. From this source the glucoside of daidzein (4′,7-dihydroxyisoflavone) has been isolated and identified as a precursor of equol. Both equol and daidzein were characterized as monoglucuronide conjugates in human urine and the concentration of urinary equol exceeded the concentrations of the classical oestrogens by 100- to 1000-fold after ingestion of a single meal containing soya protein. The potential biological significance of this result is discussed.
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INTRODUCTION

Equol (7-hydroxy-3-(4′-(hydroxyphenyl)-chroman) is an isoflavan possessing weak oestrogenic activity, in the order of 10^{-3} times that of oestradiol (Braden, Hart & Lamberton, 1967; Shutt & Braden, 1968), while also being an antioestrogen in binding competitively with oestradiol to uterine cytosol receptors (Shutt & Cox, 1972; Tang & Adams, 1980). The compound was first discovered in the urine of pregnant mares over half a century ago (Marrian & Haslewood, 1932) and then in goat (Klyne & Wright, 1957), cow (Klyne & Wright, 1959), hen (MacRae, Dale & Common, 1960; Common & Aimsworth, 1961), sheep (Braden *et al.* 1967; Shutt & Braden, 1968) and rat (Axelson & Setchell, 1981). In these animals equol is formed by intestinal bacterial degradation of phyto-oestrogens present in the feed (Batterham, Hart, Lamberton & Braden, 1965; Nilsson, Hill & Davies, 1967; Shutt & Braden, 1968; Batterham, Shutt, Hart *et al.* 1971; Axelson & Setchell, 1981). Ingestion of larger quantities of clover, particularly *Trifolium subterraneum*, which has a high content of equol precursors, leads to an infertility syndrome in sheep referred to as clover disease, in which a cystic condition in the reproductive tract is accompanied by a failure to conceive (Bennetts, Underwood & Shier, 1946; Moule, Braden & Lamond, 1963; Morley, Axelsen & Bennett, 1964; Shutt, 1976).

The occurrence of equol in the urine of man was only recently reported (Axelson, Kirk, Farrant *et al.* 1982). The amounts excreted were similar to the endogenous oestrogens, but were not related to any hormonal status.

The dietary origin of equol in man is not known, but here we report that soya protein has a remarkably high content of an equol precursor which has been isolated and identified as the glucoside of the isoflavone, daidzein (4′,7-dihydroxyisoflavone). This phyto-oestrogen is converted into equol, conjugated with glucuronic acid and is then excreted in urine.

MATERIALS AND METHODS

Urine samples

Urine (24 h) collections were obtained from a healthy man (age 34 years) and woman (age 25 years) and from 20 mature female rats (~200 g) of the Sprague–Dawley strain. The urine was collected in

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polyethylene flasks, frozen immediately and stored at −20 °C until analysed.

Diets

Rats were fed commercial pelleted food (Astra-Ewos, Södertälje, Sweden) or a semisynthetic diet, D7 (Midtvedt & Gustafsson, 1981), composed of wheat starch, casein, arachis oil, salts and vitamins. Food constituents (2–5 g/24 h) and equivalent amounts of extracts were tested for the presence of equol precursors by adding them to the semisynthetic diet in exchange for starch or oil and feeding it to one to five rats for 2 days. Soya flour (Soyolk; Soya Foods Ltd) was obtained from A/B Risenta, Stockholm, Sweden (composition: 40% protein, 20% fat and 20% carbohydrate). The two human subjects were given lunch meals in which 40 g of commercial textured soya (Natural Protoveg; Direct Foods Ltd, Petersfield, Hants; composition: 52% protein, 1% fat and 31·5% carbohydrate) was substituted for meat over a 5-day period. The soya was cooked according to the manufacturer's instructions.

Isolation of daidzein from soya

Soya flour (5 g) was refluxed for 1 h with 125 ml 80% aqueous ethanol. After filtration and evaporation of the alcohol, non-polar lipids were removed by first washing the aqueous extract with hexane (30 ml) and then passing it through a column bed (4 × 0·8 cm) of Lipidex 1000 (Packard Instrument Co., Downers Grove, Illinois, U.S.A.) (Dyfverman & Sjövall, 1978) in water. The aqueous effluent (about 30 ml) was then extracted with a Sep-Pak C_{18} cartridge (Waters Associates Inc., Milford, Maryland, U.S.A.) which was washed with 10 ml water before elution with 8 ml methanol (Shackleton & Whitney, 1980). The eluate was passed through a column bed (4 × 0·4 cm) of the strong cation exchanger sulphohydroxypropyl Sephadex LH-20 (SP-LH-20, H^+) (Axelson & Sjövall, 1979) and the material in the methanolic effluent (13 ml) was separated into neutral and phenolic fractions on a column bed (4 × 0·4 cm) of the strong anion exchanger triethylaminohydroxypropyl Sephadex LH-20 (TEAP-LH-20, OH^-) (Axelson, Sahlberg & Sjövall, 1981). Phenolic compounds, eluted from the column with 8 ml CO_2-saturated methanol, were further fractionated by preparative thin-layer chromatography (TLC) on a Merck precoated plate (silica gel 60 F_{254}; 20 cm × 20 cm, Merck, Darmstadt, West Germany) with ethylene chloride : acetic acid : water (10 : 10 : 1, by vol.). Spots were located by inspection under a u.v. lamp at 254 nm or for analytical TLC by spraying with a solution of H_2SO_4 : ethanol (1 : 1, v/v) and heating at 80–100 °C. Major components were scraped from the plate, eluted and rechromatographed on TEAP-LH-20 for removal of TLC impurities. After hydrolysis with β-glucosidase (Emulsin; Sigma Chemical Co., St Louis, Missouri, U.S.A.; 25 units in 5 ml 0·1 M-acetate buffer, pH 5, at 37 °C for 24 h), deconjugated compounds were extracted using a Sep-Pak C_{18} cartridge as described above.

Extraction and isolation of equol from urine

Equol was extracted from urine (1–6 ml) with a Sep-Pak C_{18} cartridge (Shackleton & Whitney, 1980) and conjugates, eluted with 8 ml methanol, were hydrolysed with *Helix pomatia* juice (Reactifes IBF Soc. Chim., Pointet Girard, Villeneuve la Garenne, France; 30 000 Fishman units β-glucuronidase in 5 ml 0·2 M-acetate buffer, pH 4·5) at 62 °C for 1 h (Scholler, Métay, Herbin & Jayle, 1966). After extraction on a Sep-Pak C_{18} cartridge, liberated equol in 8 ml methanol was purified by passage through a column bed (4 × 0·4 cm) of SP-LH-20 (H^+) (Axelson & Sjövall, 1979) and chromatography on a column (4 × 0·4 cm) of TEAP-LH-20 (OH^-) (Axelson *et al.* 1981). A phenolic fraction was obtained by elution of the latter column with 8 ml methanol saturated with CO_2. After removal of the CO_2 by application of vacuum, water was added to give a final concentration of 72% methanol and the sample passed through a column bed (2 × 0·4 cm) of diethylaminoethyl (DEAE)–Sephadex (Pharmacia, Uppsala, Sweden) in base form (Axelson *et al.* 1982). Monophenolic compounds were eluted with 10 ml 72% aqueous methanol and equol and other diphenolic compounds with 5 ml 72% aqueous methanol saturated with CO_2.

Isolation of equol and daidzein glucuronides from human urine

Urine (20 ml) collected from a male subject after 2 days on a soya diet was extracted on a Sep-Pak C_{18} cartridge, filtered through a column bed (4 × 0·4 cm) of SP-LH-20 (H^+) as described above and fractionated on a column bed (6 × 0·4 cm) of TEAP-LH-20 in OH^- form. After elution of monoglucuronides of neutral compounds with 20 ml 0·8 M-acetic acid in 72% aqueous methanol, glucuronide conjugates possessing a free phenolic group were eluted with 15 ml 0·4 M-formic acid in 72% methanol (Sahlberg, Axelson, Collins & Sjövall, 1981). An aliquot of the material in this fraction was analysed by TLC as for soya. R_F values of the glucuronides of equol and daidzein were 0·28 and 0·23 respectively (for comparison, oestriol 16α-glucuronide had an R_F value of 0·29). The carboxyl group of the glucuronic acid was methylated with diazomethane and the methyl esters were dissolved in methanol and purified on a column bed (4 × 0·4 cm) of TEAP-LH-20. After washing with 5 ml

methanol the conjugated equol and daidzein derivatives (which possess a free phenolic group) were eluted with 8 ml CO_2-saturated methanol.

Preparation of derivatives for gas chromatography (GC)–mass spectrometry (MS)

Methyl esters

The dried extract was dissolved in 1 ml diethyl ether: methanol (9 : 1, v/v) and diazomethane (freshly prepared by the reaction between N-methyl-N-nitroso-toluenesulphonamide and aqueous KOH, in diethyl ether; Schlenk & Gellerman, 1960) was added to the sample through a stream of nitrogen. After 30 min in an ice bath, excess diazomethane and solvents were removed under a stream of nitrogen.

Trimethylsilyl (TMS) ethers

Trimethylsilyl ethers were prepared by addition of 100 µl pyridine : hexamethyldisilazane : trimethylchlorosilane (3 : 2 : 1, by vol.) and heating at 60 °C for 30 min. The reagents were removed under a stream of nitrogen and the derivatives dissolved in hexane.

Deuterium-labelled TMS ethers were prepared by reaction with 100 µl [$^2H_9$]trimethylchlorosilane (Merck Sharp & Dohme Canada Ltd, Montreal, Canada) : pyridine (1 : 18, v/v) at 20 °C for 30 min.

Gas chromatography and mass spectrometry

Gas chromatography

Gas chromatography was carried out on a Pye 104 gas chromatograph equipped with a flame ionization detector and housing a 20 m × 0·3 mm open-tubular glass capillary column coated with SE-30 (Orion Analytica, Espoo, Finland). Nitrogen was the carrier gas with an inlet pressure of 50 kPa, giving a flow rate of about 1 ml/min. The oven temperature was 250 °C.

Quantification of equol present in the hydrolysed urine fractions was obtained by comparison of its GC peak area with that given by a known amount of authentic equol, having a retention time as a TMS ether derivative of 0·45 relative to that of 5α-cholestane. The limit of detection corresponded to about 1 µg equol in a 24-h urine collection.

Gas chromatography–mass spectrometry

Gas chromatography–mass spectrometry was done on a modified LKB 9000 instrument housing an open-tubular glass capillary column (25 m × 0·3 mm) coated with SE-30, heated at 250 °C and connected to the ion source by a single stage adjustable jet separator (Axelson & Sjövall, 1977).

Derivatized conjugated compounds were analysed on a 1·5% SE-30 packed column (1 m × 3·4 mm) at 250 °C. Temperatures of the molecular separator and the ion source were 275 and 290 °C respectively; energy of bombarding electrons, 22·5 eV, ionizing current, 60 µA and accelerating voltage, 3·5 kV. Repetitive magnetic scanning (usually six to ten scans/min) over the range of mass/charge ratios (m/z) 0–800 daltons per unit electronic charge was initiated after a suitable delay from the time of sample injection. Methods for the computerized evaluation of the mass spectral data have been described (Axelson, Cronholm, Curstedt *et al.* 1974).

RESULTS

Dietary origin of equol

Since rats excrete equol in urine and bile (Axelson & Setchell, 1981) this animal was used as a model for man in the experiments screening for dietary precursors. Evidence that commercial pelleted food contains precursors of equol was obtained by changing a normal rat feed to that of a semisynthetic diet. A marked and rapid decrease in the urinary excretion of equol to less than 2 µg/24 h was followed by an increase after the diet reverted to pellets (Fig. 1). In the subsequent search for equol precursors, different food constituents and food extracts were added to this semisynthetic diet and the excretion of equol in urine was monitored. Of the variety of food-stuffs tested, which included soya flour, soya oil, wheat, rye, oat, millet, barley, buckwheat, corn, alfalfa, white beans and brown beans, soya flour provided the richest source of precursor(s) and resulted in the urinary excretion of about

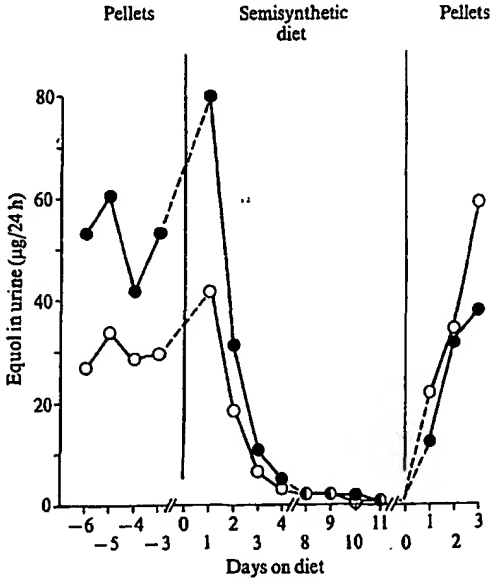

FIGURE 1. Daily urinary excretion of equol by two female rats fed commercial pelleted food or semisynthetic diet.

100 μg equol/g flour ingested (range 60–130 μg/g in five rats). The corresponding value for soya oil was only 5 μg/g. Little or no increase in the urinary excretion of equol (<1 μg/g) was observed with the other food constituents tested. Commercial food pellets contain a proportion of soya cake sufficient to account for the urinary excretion of equol by rats.

Extending these studies to man, the urinary excretion of equol in two subjects (male and female), which is normally in the range of the classical oestrogens (Adlercreutz, Fotsis, Heikkinen *et al.* 1982; Axelson *et al.* 1982), increased 100- to 1000-fold to about 4–6 mg/24 h after ingestion of 40 g soya/day (Fig. 2). Thus the response to this diet was analogous to that observed in rats, indicating that the precursor–product relationships are similar in man and rats.

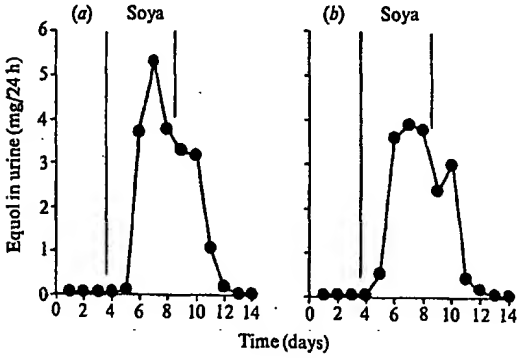

FIGURE 2. Daily urinary excretion of equol by (*a*) a man and (*b*) a woman before and after the addition of textured soya (40 g/day) to the normal diet.

Identification of daidzein, a precursor of equol in soya

Experiments to isolate the precursor(s) from soya flour showed that the compound(s) was extractable with 80% aqueous ethanol. After the fractionation of the extracted material into neutral and phenolic compounds on the ion exchanger TEAP-LH-20, approximately 90% of the precursor(s) was present in the phenolic fraction. Preparative TLC showed a major component of R_F 0·32. When this component was fed to rats it was readily converted to equol and quantitatively accounted for approximately 75% of the expected excretion of equol from the original amount of soya. The TMS ether of this compound, when analysed by GC–MS, did not show the molecular ion (M) at *m/z* 776 (Fig. 3) but ions at *m/z* 761 (M-15), 671 (M-(15+90)) and 581 (M-(15+2×90)), which are formed by loss of a methyl group from the molecule and subsequent loss of one and two derivatized hydroxyl groups respectively. The base peak at *m/z* 361 and the ions of *m/z* 271, 243, 217 and 204 are indicative of a TMS ether of a hexose structure (Laine & Elbein, 1971). In analogy with persilylated glucuronide conjugates (Spiegelhalder, Röhle, Siekmann & Breuer, 1976) the ion at *m/z* 450 indicated that the sugar residue was conjugated to an aromatic hydroxyl group. This ion arises from the glycone with a loss of a proton. Aromatic conjugation was further supported by the intense ion at *m/z* 398 (M-378), which is formed by deconjugation and transfer of a TMS group from the glycoside moiety to the aglycone (Billets, Lietman & Fenselau, 1973; Spiegelhalder *et al.* 1976). The ion at *m/z* 383 is formed by subsequent loss of a methyl group from the aglycone.

Treatment of this compound with β-glucosidase yielded a product (R_F value 0·83 compared with 0·92 for equol on TLC), the TMS ether derivative of which had an identical GC retention time on SE-30 (1·12 relative to 5α-cholestane) and mass spectrum as the persilylated authentic daidzein. The mass spectrum showed a molecular ion and base peak at *m/z* 398 (Fig. 3). The origin of ion *m/z* 355 is unknown, it may be due to loss of CH_3 (*m/z* 383) and CO (Budzikiewicz, Djerassi & Williams, 1964). The fragment ion at *m/z* 190 may represent $((CH_3)_3SiO\text{-}C_6H_4-C{\equiv}CH)^+$ in analogy with the fragmentation of equol (Axelson *et al.* 1982). These interpretations were supported by the analysis of the compound as a perdeuterated derivative.

The glycoside moiety of the daidzein conjugate occurring in soya was tentatively identified as a glucose residue. This is based on the following properties and evidence: hydrolysis with β-glucosidase, mobilities on the anion exchanger TEAP-LH-20, TLC and GC (retention time on SE-30 was 19 times that of the TMS ether of daidzein), the mass spectrum and the previous finding of daidzein glucoside in soya beans (Walz, 1931). Definite confirmation that daidzein is the major precursor of equol in commercial soya flour was obtained by feeding a rat the reference compound daidzein (acetate, 400 μg) in the semisynthetic diet, which yielded approximately 50 μg equol in the urine.

Identification of equol and daidzein glucuronides in human urine

In our earlier characterization of equol in urine (Axelson *et al.* 1982) it was tentatively identified as a glucuronide conjugate, consistent with the majority of endogenous urinary steroids. After repetitive scanning MS of the GC-effluent, the presence of equol and daidzein glucuronides was evident from the reconstructed chromatograms for diagnostically significant ions given by the methyl ester TMS ether derivative of the two compounds (Fig. 4). Peaks occurred at 14 and 32 min (retention time of 5α-cholestane was about

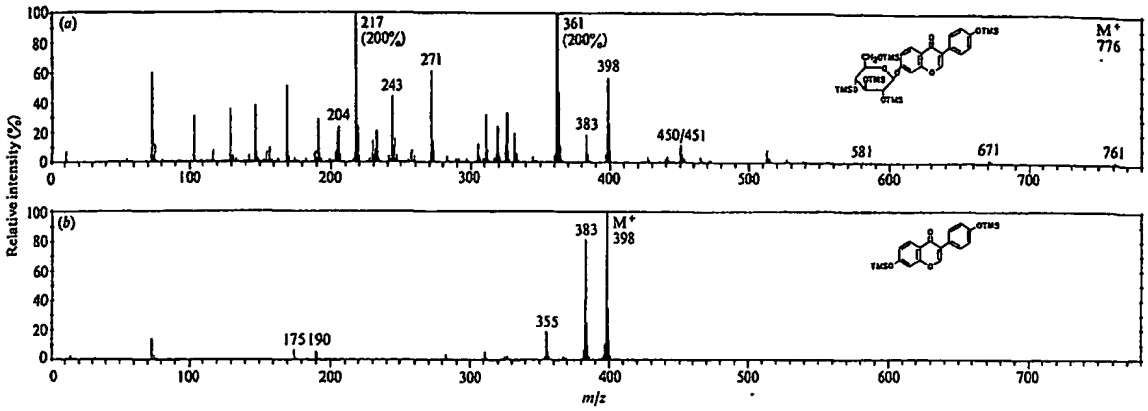

FIGURE 3. Electron impact ionization mass spectra of the trimethylsilyl (TMS) ethers of daidzein (4',7-dihydroxy-isoflavone) glucoside isolated from soya flour (*a*) before and (*b*) after treatment with β-glucosidase. M^+, molecular ion; *m/z*, mass/charge ratio in daltons per unit electronic charge.

1 min) which represent intact glucuronides of equol and daidzein respectively. The complete mass spectrum of equol glucuronide eluted at 14 min is shown in Fig. 5. Consistent with the mass spectra of derivatives of glucuronide conjugates of oestrogens (Spiegelhalder *et al.* 1976) the relative intensities of the molecular ion (*m/z* 720) and the ions formed by losses of a methyl group (*m/z* 705) and TMS groups (*m/z* 615 and 525) are below 5% in this aromatic conjugate. The loss of 292 and 334 mass units from the molecular ion has previously been observed in mass spectra of phenolic glucuronide conjugates (Billets *et al.* 1973; Spiegelhalder *et al.* 1976; Axelson & Setchell, 1980); the latter fragmentation represents the loss of glucuronic acid with the corresponding transfer of a TMS group to equol. The ion at *m/z* 192 consists of a derivatized phenol group with a 2-carbon chain which is the base peak in the mass spectrum of unconjugated equol (Axelson *et al.* 1982). Ions at *m/z* 406, 407, 317 (base peak), 275, 217 and 204 are all typical of the fragmentation of the glucuronic acid (Billets *et al.* 1973; Spiegelhalder *et al.* 1976; Axelson & Setchell, 1980).

The glucuronide conjugate of daidzein was also identified in the same urine sample (Fig. 4). The mass spectral fragmentation pattern of the methyl ester TMS ether of the intact conjugate (Fig. 5) was similar

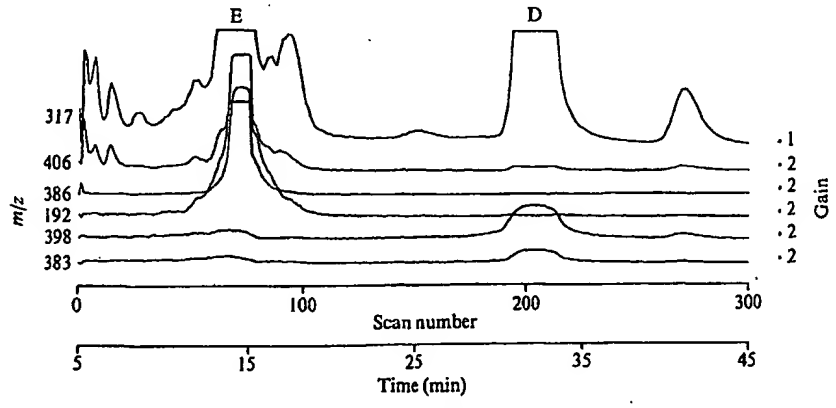

FIGURE 4. Gas chromatographic–mass spectrometric analysis of methyl ester trimethylsilyl ether derivatives of intact glucuronides of equol (E; 7-hydroxy-3-(4'-hydroxyphenyl)-chroman) and daidzein (D; 4',7-dihydroxy-isoflavone) isolated from human urine. Fragment ion current chromatograms of mass/charge ratio (*m/z*) 317 daltons per unit electronic charge and *m/z* 406 are representative of the glucuronyl moiety, those of *m/z* 386 and *m/z* 192 of equol and those of *m/z* 398 and *m/z* 383 of daidzein structure (see text). For purpose of illustration the intensities of *m/z* 317 were multiplied by a factor of 1, the other ions by a factor of 2.

to that of equol glucuronide. The loss of glucuronic acid in a rearrangement with the simultaneous transfer of a TMS group to the aglycone gives rise to the significant ion at *m/z* 398 (M-334) which is the molecular ion and base peak in the mass spectrum of the TMS ether of daidzein (Fig. 3).

These data firmly establish the occurrence of equol and daidzein in human urine as glucuronide conjugates. However, it is not possible to determine the position of conjugation by these methods, and the presence of two isomers of each compound cannot be excluded.

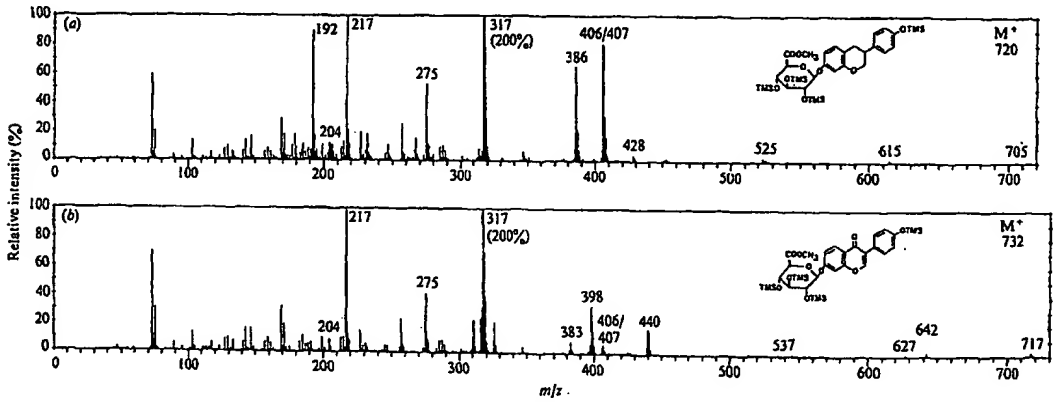

FIGURE 5. Mass spectra of the methyl ester trimethylsilyl (TMS) ether derivatives of the glucuronides of (*a*) equol (7-hydroxy-3-(4'-hydroxyphenyl)-chroman) and (*b*) daidzein (4',7-dihydroxy-isoflavone) isolated from human urine. The origin of ions is given in the text. M^+, molecular ion; *m/z*, mass/charge ratio in daltons per unit electronic charge.

DISCUSSION

As shown here, and in a subsequent study (Setchell, Borriello, Hulme *et al.* 1984), soya meal is a major dietary source of urinary equol in man. Equol itself was not detected in soya but was shown to be formed from the glucoside of daidzein. Glucosidases are known to be present in intestinal bacteria (Drasar & Hill, 1974), which have also been shown to carry out the reduction and deoxygenation reactions required for conversion of daidzein to equol in animals (Batterham *et al.* 1965; Nilsson *et al.* 1967; Batterham *et al.* 1971) and recently in man (Setchell *et al.* 1984). Germfree rats, however, do not excrete equol when given commercial pelleted food (Axelson & Setchell, 1981). These results strongly suggest that also in man equol is formed in the gastrointestinal tract as a result of the bacterial degradation of daidzein. Thus, the rate of formation of equol from daidzein is conceivably influenced by the composition of the microflora, the intestinal transit time and the redox level in the large intestine. These conditions are affected by the diet, and when rats were fed the semisynthetic diet for several weeks the capacity to form equol decreased or disappeared in several rats. When pelleted food was again given, the capacity was partly regained. A variable yield of urinary equol has also been observed in man after ingestion of soya (Setchell *et al.* 1984). Conjugation of equol and daidzein with glucuronic acid most likely occurs in the liver as is the case with most endogenous oestrogens.

Although daidzein was the only equol precursor identified from soya, other precursors may exist in plants and foods. Isoflavones such as formononetin (7-hydroxy-4'-methoxyisoflavone), biochanin A (5,7-dihydroxy-4'-methoxyisoflavone) and genistein (4'-5,7-trihydroxyisoflavone) are all potential precursors of equol in animals (Cayen, Carter & Common, 1964; Batterham *et al.* 1965, 1971; Nilsson *et al.* 1967; Shutt & Braden, 1968; Tang & Common, 1968; Batterham *et al.* 1971). Soya beans can contain an abundance of phyto-oestrogens (Walz, 1931; Walter, 1941; Naim, Gestetner, Kirson *et al.* 1973; Lookhart, Jones & Finney, 1978), particularly genistein and daidzein, which have been ascribed to cause uterotrophic effects in laboratory mice given soya bean meal (Carter, Smart & Matrone, 1953; Cheng, Story, Yoder *et al.* 1953) or commercial pelleted food (Drane, Patterson, Roberts & Saba, 1975, 1980). Our observations here and earlier that equol is the major phenolic compound found in urine, blood and bile of rats maintained on this diet (Axelson & Setchell, 1981) suggest that the oestrogenic effects are more likely to be induced *in vivo* by equol than by genistein and/or daidzein. Genistein glucoside was not detected in the commercial soya flour used in the present study. Whether this is due to variations in the composition of isoflavones between different species of soya beans or due to elimination of

genistein during the manufacture of the flour is not known.

The widespread use of soya beans as a protein food source makes it important to determine possible physiological effects of equol in man. The 'contraceptive' effect in animals suggests to us that it may be of interest to investigate the dietary habits and urinary excretion of equol in women with unexplained infertility or disorders of the menstrual cycle.

In addition, whether the presence of phytoestrogens and related oestrogenic compounds in common food-stuffs affect the development and/or treatment of hormone-dependent tumours should also be considered.

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Western diet and Western diseases: some hormonal and biochemical mechanisms and associations

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Breast cancer, prostate cancer, coronary heart disease and colon cancer belong to the so-called Western diseases and a general opinion is that diet is a significant or even the main factor increasing incidence and mortality of these diseases in the Western world. This review describes studies carried out in this department for about 10 years, many in collaboration with scientists abroad, and with the aim to clarify some of the connections between the diet and sex hormone, lipid and bile acid metabolism. A Western-type diet elevates plasma levels of sex hormones and decreases the sex hormone binding globulin concentration, increasing the bioavailability of these steroids. The same diet results in low formation of mammalian lignans and isoflavonic phytoestrogens. These diphenolic compounds seem to affect hormone metabolism and production and cancer cell growth by many different mechanisms making them candidates for a role as cancer protective substances. The precursors of these diphenols are to be found in fiber-rich unrefined grain products, various seeds, beans and probably also in pulses, peas and berries. Some types of fiber seem to influence sex hormone and bile acid metabolism mainly by partial interruption of the enterohepatic circulation, by alteration of intestinal metabolism and by increasing fecal excretion of these compounds. The sex hormone pattern found in connection with a Western-type diet is prevailing in the breast cancer patients, but is only partly a result of the diet.

Key words: breast cancer, prostate cancer, colon cancer, coronary heart disease, diet, fiber, lignans, isoflavones, estrogens, androgens, sex hormone binding globulin, dihydrotestosterone, bile acids, feces

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Breast cancer (BC), prostate cancer (PC) and endometrial cancer (EC) belong to the group of hormone-dependent cancers which in addition to colon cancer (CC), coronary heart disease (CHD) and some other diseases are called Western diseases because their incidence and mortality are high in the Western world compared to countries in Asia and South and East Europe [1–3]. In migrant studies an increased risk for Western diseases has been found to be related to a change towards a Westernized diet [4–9]. Migrants from Asia, Africa or East Europe to U.S.A. or Australia have

originally consumed a low-fat vegetarian or semi-vegetarian diet containing large amounts of unrefined carbohydrates. Most of these migrants and their children rapidly adopt a diet rich in calories, fat and proteins and low in complex carbohydrates and fiber [10] and their hormone [11, 12] and lipid levels change towards a Western pattern, increasing the risk for hormone-dependent cancer and CHD. Interestingly, migrants from a high risk colon cancer area (Scotland) to Australia experience a reduced risk for colon cancer [9].

Furthermore Hill et al. [13] postulated that a Western-type diet increases the concentration and metabolism of fecal bile acids (FBA) and neutral sterols (FNS), increasing the risk for CC. In the majority of the population studies carried out this hypothesis has rendered support with regard to the concentration but not with regard to the metabolism of FBA and FNS [review in 14]. On the other hand many animal experiments and *in vitro* tests have shown that free bile acids are cocarcinogenic or comutagenic [15, 16] but that the aminoconjugated bile acids may be inactive [16] in this regard [reviews in 14, 17]. It is believed that secondary bile acids are more toxic than primary ones and that a high litocholic acid (LCA) to deoxycholic acid (DCA) ratio is a CC risk factor [18–20].

Because of the obvious relationship between Western diet and Western diseases it has been postulated that this type of diet by some biochemical or other mechanisms may alter hormone production, metabolism or action at the cellular level increasing the risk for hormone-dependent cancer. Furthermore it has been suggested that the dietary composition may influence transit time of the intestinal content, fecal bulk and intestinal microflora and its environment causing alterations in concentration and metabolism of hormonal steroids, bile acids, neutral sterols, carcinogens and procarcinogens increasing the risk of CC and BC. Particularly in women, who have a much higher incidence of hormone-dependent cancer than men, diet has been suggested to be the main single determinant in the etiology of these cancers.

It is, however, very difficult to separate the effects of various single macro- or micronutritients on any biochemical event or steroid hormone or bile acid pattern or level. This is not only due to difficulties in the accurate recording of the diet, but also to the great variability in dietary intake during different seasons and even different parts of the week and the variability of hormone and steroid levels, particularly in women. Special efforts have to be made to standardize the conditions for sampling and to use reliable hormone assay methods and the recording of the diet must be carried out during sufficiently long time [12].

The following review will summarize and discuss results of our studies on the connection between diet and Western diseases. Many of these investigations are the result of collaborations with scientists abroad and some results discussed have not yet been published. The review will deal with some newly discovered mechanisms of dietary effects on sex hormone and intestinal bile acid metabo-

TABLE I. Abbreviations and trivial names of steroids and other abbreviations used in the text.

A	Androstenedione
BC	Breast cancer
CC	Colon cancer
CHD	Coronary heart disease
DHEAS	Dehydroepiandrosterone sulfate
Da	Daidzein
DCA	Deoxycholic acid
5α-DHT	5α-Dihydrotestosterone
EC	Endometrial cancer
End	Enterodiol
Enl	Enterolactone
E2	Estradiol
E3	Estriol
E1	Estrone
E1S	Estrone sulfate
Eq	Equol
FBA	Fecal bile acids
FNS	Fecal neutral sterols
For	Formononetin
FE2	Free estradiol
FT	Free testosterone
Gen	Genistein
2-OHE1	2-Hydroxyestrone
4-OHE1	4-Hydroxyestrone
%FT	Percentage free testosterone
%FE2	Percentage free estradiol
PC	Prostate cancer
LCA	Litocholic acid
LH	Luteinizing hormone
Mat	Matairesinol
SHBG	Sex hormone binding globulin
T	Testosterone

In his recent review Zumoff [81] includes nine hormone-related hypotheses in the discussion on hormones and BC, but none of them was discussed in relation to diet despite the huge amount of epidemiological data suggesting that a Western diet plays an essential role in increasing the BC risk in the Western world.

Because of the extensive literature I will discuss only a few of those hypotheses regarding the association of sex hormone alterations and BC, which seem to be related to diet.

The main change in diet when subjects from developing countries migrate to Western countries is an increase in animal fat and protein and a decrease in intake of complex carbohydrates, particularly whole grain products [10]. This change is identical to what has occurred in Scandinavia in the last 300 years and in fact has been going on in Finland since World War II with a simultaneous increase in the incidence of BC, CC and other Western diseases. I therefore like to discuss particularly the possible role in cancer development of complex carbohydrates like whole grain products and soy beans, cereal fiber and the role of lignans and isoflavonic phytoestrogense and their association with plasma SHBG and the % free sex hormones.

In two case-control [82, 83] and in an epidemiological study [84] it was shown that high fiber and high carbohydrate intake, respectively, decreased the risk of BC. In another case-control study particularly fiber from grains consumed during adolescence reduced the risk both in premenopausal and postmenopausal women [85]. These observations are in agreement with the results of our studies in postmenopausal women in Boston [35] and in premenopausal women in Helsinki [41] showing that the main and in fact only really significant difference between the diet of the BC patients and the omnivorous and vegetarian control women was a low intake of grain products and grain fiber. If the diets of the Boston and Finnish women studied by us are compared, the main difference is also in the grain and grain fiber intake, being much higher in the Helsinki women with a lower risk for BC than the Boston women. This dietary difference caused the mean fecal weights to be higher in the Finnish compared to the Boston women, despite similar mean total fiber intakes. The large fecal bulk affects the enterohepatic circulation of sex hormones, because there is *e.g.* a significant correlation between fecal weight and fecal estrogens. In both countries the fat/fiber ratio was the same in the omnivorous and BC women, but much lower in the vegetarian women, particularly in Boston, because the Finnish vegetarians consumed rather much fatty milk products. The postmenopausal Boston BC women had lower fat intake than the Finnish young vegetarians (!), the protein intake being similar. However, the fat to grain fiber ratio (g/g) was 16.4 in the old Boston BC women and only 10.2 in the young Finnish BC women and the corresponding values for the omnivores were 15.1 and 8.2, respectively. The Boston and Helsinki vegetarians had total fat/grain fiber ratios of 7.1 and 6.3, respectively. Very interesting are also the results of the protein/grain fiber (g/g) ratios in the six groups of women, The vegetarians, omnivores and BC patients in Boston and Helsinki had the following ratios: 7.2, 15.2, 18.1, and 5.4, 7.2 and 8.8, respectively. This shows that these ratios are very high in the omnivorous women and the BC patients in Boston, and also highest in the BC group in Helsinki compared with the other Finnish women mainly due to differences in grain fiber intake.

The fat intake in the BC women both in Boston and Helsinki was intermediate between that of the omnivores and vegetarians in respective city. This may be due to bias, particularly in Helsinki, because of much propaganda in this country about reducing fat intake in order to avoid cancer and other diseases. However, small differences in fat intake will not have any detectable effect on plasma or urinary sex hormone levels (for discussion see [12]), which may to some extent explain the results of a recent prospective study [86] in nurses that failed to show any correlation between high fat consumption and the subsequent development of BC (see also [87, 88]. However, in our opinion, after considering our above-mentioned results, it seems more appropriate to use the fat/total fiber or fat/grain fiber ratio to define the diet of risk groups and controls than to use % fat calories or total fat intake. However, recent prospective studies in our laboratory suggest that

particularly grain products containing all compounds of the grain may be protective and that so-called whole-meal products may be less satisfactory in this sense (see below). Also protein/carbohydrate or particularly protein/total fiber or protein/grain fiber ratio should perhaps be used to define the dietary groups. Using such ratios we have observed that the association of diet to sex hormone metabolism becomes much more obvious. We believe that this is related to the intestinal metabolism of hormones, lignans and isoflavones, which is dependent on the intestinal environment and closely related to our diet and perhaps better described or reflected by these ratios than by expressing the amounts of macronutritients as percentages of total calories or in relation to body weight.

Without doubt it is not fat alone wh
negative effects on overall sex hormone
proteins, fiber and complex carbohydrat
least in Western societies to play even m
tial roles. As an example of what this
means is the increasing effect of a high fat
and low grain intake both in man and in
mental animals on intestinal β-glucuronid
literature in [21, 23]), which theoretically l
an increase of the reabsorption of estrogen
the intestinal tract [25] and higher plasma estrogen levels [23, 24]. It should also be emphasized that the associations between fiber intake and the excretion of a number of urinary estrogens became statistically significant first when the fiber/kg body weight ratio was used instead of total fiber intake [26]. The fiber/kg ratio may better reflect the intestinal bacterial environment and fiber effects because a small subject has a smaller "internal" volume of the intestines compared to a tall subject.

The diet in Finnish rural areas where BC and CC incidence is low differs from the American one particularly with regard to its relatively high content of complex carbohydrates mainly from whole-grain products and starchy vegetables, the fat content being similar but deriving more from milk products than from meat [89, 90 and own observations]. A significant part of the Finnish milk product consumption consists of fermented milk products. Because of the differences in BC risk in USA and Finland we have postulated that this difference is at least partly due to the great difference in intake of whole-grain fiber-rich products like rye bread and perhaps some other fiber-rich nutritients such as berries. Particularly these foodstuffs increase the excretion of urinary lignans by the Finns and affect simultaneously also otherwise the intestinal milieau. This view was supported by the finding of very low urinary lignan excretion in the BC subjects living in Boston [57] and of lower excretion also in the young BC women in Helsinki [34, 73]. In both BC groups it was likely that the differences were due to low intake of whole-grain products. However, in Helsinki the differences between the omnivorious, vegetarian and BC groups were relatively small, because the grain intake was comparably high in all groups, which

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published) seem to significantly increase lignan excretion in Finnish women. This is because during modern milling of the grain, trying to eliminate so-called antinutritional factors [76], simultaneously also the diphenolic plant lignans seem to be at least partly eliminated. There are indications that also berries, fruits and various seeds [33, 56, 91] increase lignan excretion. Of some grain products, rye meal seems to result in the highest excretion of lignans in rats, followed in decreasing order by oat, barley and wheat meal [91]. The latter results are difficult to evaluate because no exact details were presented regarding the nature of the meal products consumed by the rats.

Based on an epidemiological study it was recently suggested that consumption of fermented milk products may protect against breast cancer [92]. In a case-control study consumption of fat from milk, cheese and yogurt during adolescence reduced the BC risk both in premenopausal and postmenopausal women [85]. One mechanism by which fermented milk may influence hormone metabolism

is by reduction of the β-glucuronidase-producing bacteria of the intestinal content [93, 94], which theoretically should reduce the enterohepatic circulation of estrogens and increase the fecal route of elimination. The conjugated estrogens excreted in the bile must be deconjugated before the estrogen moiety can be reabsorbed. Milk products have also been found to contain animal lignans and isoflavonic phytoestrogens (75) and even if the concentrations are rather low they add to those produced by the intestinal bacteria from plant precursors.

Our hypothesis has been that high intake of whole-grain products (preferably in combination with reduced fat and moderate protein intake) reduces BC (and CC) risk because such a diet increases fecal bulk and reduces intestinal β-glucuronidase activity and steroid and bile acid enterohepatic circulation and results in increased mammalian lignan production [12, 21]. Later on we also included the isoflavonic phytoestrogens into the original theory [33, 54]. This was due to the finding of very high excretion of isoflavonic phytoestrogens in urine of Japanese men and women consuming a traditional diet [33, 72]. The lignan excretion in the Japanese subjects was low, even lower than we found in the postmenopausal BC patients in Boston. The isoflavones resemble lignans with regard to structure (all are diphenolic). In most correlation studies they show parallel behaviour. In the Finnish women the significances of the positive correlation between the excretion of lignans and isoflavonic phytoestrogens in urine, and plasma SHBG, and the negative correlations with %FE2 and %FT are stronger than the separate correlations for each group of compounds [33]. Recently, our hypothesis with regard to the protective role of these compounds for BC got strong support from studies showing that powdered soy bean chips, both before and after denaturation of protease inhibitors, decrease mammary tumor formation in a rat breast cancer model [95]. Furthermore Gen, found by us in human, chimpanzee and cow urine, may be anticarcinogenic due to its inhibitory effect on protein tyrosine kinase [61–64] and other flavonoids are antiproliferative with regard to BC cells [59]. The postmenopausal BC patients in Boston had the lowest plasma SHBG and highest %FT and %FE2 [35] and the lowest Enl and Eq excretion [57]. The Finnish premenopausal BC subjects had lower SHBG, higher %FT and %FE2 and lower excretion of lignans and isoflavonic phytoestrogens compared to the vegetarians [34]. In many studies low SHBG has been associated with BC (see literature in [35, 96]).

Because of the large differences in grain fiber intake and urinary lignan excretion between postmenopausal women living in Helsinki and Boston we have in preliminary calculations combined the materials of postmenopausal women and found the same highly significant positive correlation between grain fiber intake or Enl excretion and plasma SHBG and negative correlations with plasma %FE2 and FT (unpublished observations) as we found for the young Finnish women [33, 34].

The theory based on the observation that high fat intake increases 16α- and decreases 2-hydroxylation of estrogens leading to biologically more active estrogens also needs some discussion. According to this theory a low rate of 2-hydroxylation and high rate of 16α-hydroxylation leads to a greater risk for BC and endometrial cancer [52, 53, 97–99] because 2-hydroxylated estrogens are biologically less active than 16α-hydroxylated ones. Several earlier studies as well as our own seem to speak against this hypothesis because all low-risk groups, compared to high-risk groups, have relatively more urinary 16α-hydroxylated estrogens, particularly if also the fecal estrogens are included. Women living in low-risk countries consume most of their calories in the form of complex carbohydrates and have lower fat and protein intake, which should lead to low 2-hydroxylation of estrogens [38, 39]. This we could observe in the young premenopausal Finnish women [40, 41] and in the previously investigated Oriental women [23, 42]. The characteristics of the sex hormone pattern in these low-risk Oriental women on a low-fat diet are low plasma levels of E1, E2, A and T and low excretion of E1, E2 and 2-hydroxylated estrogens and relatively high amounts of both 16α- and 16β-hydroxylated estrogens [23, 42] We could also not see any increase in 16α-hydroxylated estrogen metabolites in urine of Finnish premenopuasal women with

BC. In fact slightly higher mean values were seen in the vegetarians, but the differences were not significant [40, 41].

Recently we completed the second part of the Finlandia study dealing with groups of postmenopausal women and found results apparently more in line with those suggesting that high 16α-hydroxylation is a risk factor. A statistically significant (logarithmic) negative correlation between plasma SHBG and urinary 16α-hydroxyestrone ($R = 0.59$, $p < 0.001$) and estriol ($R = 0.49$; $p < 0.01$) was found with the highest values of estriol and lowest SHBG values in the BC and omnivorous women and higher SHBG and lower urinary 16α-hydroxylated estrogens in the vegetarians. In the same material there was a significant positive correlation between urinary total diphenol excretion and plasma SHBG ($R = 0.64$; $p < 0.001$). From our results it appears that the tendency to lower values of 16α-hydroxylated estrogens in urine of the vegetarian and higher in the omnivoric and BC women is probably due to different degrees of fecal elimination of these estrogens as a result of differences in fiber intake and not to increased 16α-hydroxylation of estrogens in BC. However, the evaluation of this very large study is still in progress and the definite results have to await the extensive statistical treatment needed. In these postmenopausal women we found no correlation between plasma SHBG and urinary catecholestrogens but a highly significant positive association between the logarithms of plasma E1S and urinary excretion of 2-hydroxy-E1 ($R = 0.84$; $p < 0.001$). The BC women tended to have both higher plasma E1S and urinary 2-hydroxy-E1, which supports our theory that high E1 and E1S and urinary catecholestrogens may be risk factors of BC. It may be mentioned that high E1S has also been found in EC [100].

With regard to 2-hydroxylated estrogens there is evidence speaking for a role of these steroids and catecholestrogens formed from stilbestrol in hormonal carcinogenesis via microsome-mediated redox cycling and formation of quinones and free radicals [101]. The quinoid structures are prerequisites for the genotoxic effect [102] because they are capable of covalent binding to proteins [103, 104]. The development of renal tumors in Syrian hamsters after estrogen treatment has been postulated to occur via a free radical mechanism [105]. Hydroxylated flavonoids have antagonistic effects on the mutagenic and/or tumorigenic activity of epoxide metabolites of polycyclic aromatic hydrocarbons [106]. Because of similar structure the isoflavones and lignans should also be investigated in this respect.

DIET, HORMONES, LIGNANS AND ISOFLAVONES, AND OTHER WESTERN DISEASES

It is not possible in this connection to discuss at any length the relationships between diet and other Western diseases. Some very large reviews on nutrition and its relationship to cancer have been published [107, 108]. However, I would like to discuss shortly some new results indicating that the above discussion may have some important implications also for other diseases than BC and that obvious hormonal and biochemical connections exist between BC and other Western diseases.

Endometrial cancer

What has been said about diet and estrogen metabolism and BC holds as well for EC, a disease even more clearly estrogen-dependent than breast cancer. An increase in bioavailable estradiol due to lowering of SHBG and increase in reabsorption of biliary estrogens as a result of a Western diet would also promote the growth of endometrial cancer. This cancer type has in addition been found to be associated with other diseases common in the Western world, like hypertension and diabetes. Hypertension has in fact recently been found to be a risk factor also of BC [109].

Prostate cancer

Furthermore, it is known that a low-fat and/or high-fiber diet affects sex hormone metabolism also in men [28–30] by decreasing T and FT. A high level of biologically active androgens probably accelerates the development of PC in the Western world and recently a prospective study in fact seems to indicate that elevated T levels are

associated with increased risk of PC [110]. In epidemiological studies fat and meat show a positive and cereals a negative association with PC mortality [3]. In Japan and some other Asian countries, despite the same incidence of latent small or non-infiltrative prostatic carcinomas, the mortality is low [111–113]. This could at least partly be explained by a diet-related lowering of biologically active androgens as seems to occur in Asian women [24] and in the above-mentioned experimental studies [28–30]. Rotkin (cited from [114]) suggested that the men at risk of developing PC had a "strong overbalance of androgenic components" and observed that fewer patients with prostatic cancer developed gynecomastia and obesity early in life compared to controls. However, also recent observations indicate a possible protective effect of endogenous estrogens [115, 116] and this would suggest that the high levels of isoflavonic phytoestrogens in the traditional diet of Japanese men [33, 68] may also represent a protective factor [33, 54] inhibiting the growth of already existing small cancers [theory originally proposed in 117]. However, other than estrogenic effects of these substances may be more important.

The above-mentioned theory gains support from the recent observations of decreased risk of prostate cancer in Adventist men showing high consumption of beans, lentils, peas and some dried fruits (dietary sources of flavonoids) [118] and in men of Japanese ancestry in Hawaii consuming much rice (mainly starch, which may have some fiber-like effects in the gut) and tofu [119], a soy bean product. Our own results in Japanese men and women [some results in 72] show a strong positive association between the intake of various soy products and urinary excretion of equol and daidzein, and also a positive correlation with lignan excretion, particularly enterodiol, despite the fact that lignan excretion was low in the Japanese subjects investigated. It was in fact suggested [112], that if new small latent carcinomas are being formed at a constant rate they may either disappear or may enlarge and develop into larger carcinomas in different numbers or at different speeds in different geographical areas. It is suggested that in certain populations dietary factors affect androgen metabolism and biological activity as described above and/or that dietary isoflavones and other phytoestrogens directly influence cancer cell growth slowing the speed of development of these small latent carcinomas. The possible effect of soybean diets on PC may be a parallell to the observation of the inhibitory effect of this diet on breast tumor incidence in experimental animals [95, 120].

Coronary heart disease

Low SHBG has been found to be a risk factor of CHD mortality in a female population during a 12-year follow-up period [121] and is probably a risk factor also in men [122]. In addition, low plasma 5α-DHT seems to be a risk factor of CHD in men [122, 123]. As mentioned previously a high dietary protein/carbohydrate ratio not only suppresses plasma levels of SHBG, but simultaneously inhibits liver 5α-reductase [36–39]. Furthermore, we found significantly higher SHBG and HDL-cholesterol and almost significantly higher 5α-DHT ($p < 0.07$) in joggers compared to the subjects with CHD and a positive association between SHBG and HDL-cholesterol and maximal oxygen uptake in both joggers and healthy men [122]. Plasma SHBG and 5α-dihydrotestosterone concentration correlates positively with HDL-cholesterol and apoliprotein A-I both in healthy middle-aged men and in men with CHD [122, 123]. It is also known that thyroid hormones and estrogens stimulate SHBG synthesis, increases liver 5α-reductase and plasma HDL-cholesterol and apolipoprotein A-I [124, 125]. In population studies HDL-cholesterol and apolipoprotein A-I are inversely related to CHD [126, 127]. Compounds increasing the 5α-/5β-reductase activity ratio in rat liver microsomes lower serum cholesterol and reduces the incidence and severity of atherosclerotic lesions in aortas of cholesterol-fed rabbits [128]. Whether the higher plasma SHBG and 5α-DHT in our physically fit men compared to the subjects with CHD is due to diet or to physical exercise itself cannot be judged at present. The protein/carbohydrate ratio of the diet may be lower in hard-training joggers, which could explain the high SHBG and 5α-DHT levels. This is because aerobic training usually leads to increased proportion of carbohydrates in the diet.

In Finnish men this may mean increased consumption of whole-grain rye products, because about 40 % of the cereals consumed in Finland are rye products [see *e.g.* 129] and rye bread is usually a whole-grain product in this country.

As already mentioned consumption of whole-grain rye bread has recently been found by us to considerably increase animal lignan excretion in urine (R. Korpela, H. Adlercreutz, to be published), and it also seems to stimulate SHBG synthesis (almost statistically significant increase after 2 weeks; $p < 0.07$) as suggested previously [33]. Furthermore, it is of interest that isoflavones, excreted in high amounts in urine in populations having a low CHD risk, like the Japanese men, have hypocholesterolemic effects in rats [130] and that treatment with a soybean-protein diet has remarkable hypocholesterolemic effects in human subjects with type-II hyperliproteinemia [131]. Soybean protein products contain isoflavonic phytoestrogens, but whether the effect observed is due to these compounds or to the plant protein itself, as suggested by the authors, is uncertain. It is interesting to note that it has been suggested that the hypocholesterolemic effects of isoflavones is probably independent of the estrogenic effects [130]. Furthermore, it has been shown that the hypocholesterolemic effect of soy products in human subjects is not due to the content of soybean fiber [132]. It is concluded that very similar associations between diet, SHBG, lignans and isoflavones, as found for BC, seem to exist also with regard to CHD.

Colon cancer

In epidemiological studies a parallelism has been observed between BC and CC [133], but there are also some discrepancies suggesting different etiology [review 86]. However, for none of the Western diseases the etiology is likely to be monofactorial and looking only for the associations with macronutritients may easily lead us to wrong conclusions. There are also some parallelisms between CC and PC [3], and diet, in the majority of the opinions [107,108], seems to be the most important environmental factor in the development also of CC.

CC has also been found to be related to reproductive and hormonal factors [review in 130] and it has been found that increasing parity decreases risk and late age at first live birth increases risk [135, 136] as found also for BC. Women with cancers of the breast and other reproductive sites have an excess of primary colorectal cancer and pregnancy protects against DMH-induced colon cancer in experimental animals [review in 136]. Many colon tumors contain sex hormone receptors [137-140], and they may play a role in the pathogenesis of the disease [141].

The observed discrepancies in parallelism between CC and BC incidence and mortality development in Japan [86] may be due in addition to changed consumption of macronutritients to some micronutritients like plant lignans and isoflavones having a large spectrum of biological activities like anticarcinogenic, antiproliferative, antihormonal or hormonal and antiviral effects, which may play a role also locally in the intestine [21, 142]. The local effects in the intestine may be independent of the formation of the hormonally active substances which seem to alter liver and peripheral sex hormone metabolism. Another factor which may play a role for the discrepancies in parallelism between CC and BC is that a change in the fat content of the diet e.g. in Japan may not parallel a change in the use of soy products, because the soy sauce is mainly used for its content of sodium chloride and other soy products may still be used independently of an increase in fat intake. When leaving the habit to consume a low-fat diet the Japanese seem to still consume rice and they do not get any additional (cereal) fiber needed to compensate for the higher fat intake, because whole-grain bread seems to be almost unknown in Japan. This in our opinion could perhaps explain that the CC incidence in Japan increases more rapidly than the BC incidence [86] because of the absence of cereal fiber but continuous consumption of soybean products and rice.

Furthermore, an increase from 10 to 25 % of the fat calories as has occurred in Japan between 1955 and 1975 [86] may not alter the hormonal pattern as much as the difference we find for urinary and plasma sex hormones when the fat calory intake is

about 20 % compared with that found when it is about 38 % [24, 42]. In our own studies in a rural village outside Kyoto [72] women and men still consume only 20 and 17 % fat calories, respectively.

In most epidemiological studies a relation between fat intake and CC has been observed, but in only few studies an association has been found between CC risk and high protein intake or high energy consumption [143, 144] both leading to low SHBG, despite the fact that fat and protein consumption generally increase in parallel. In one study a high meat/vegetable consumption ratio predisposed for CC [145], a diet, which probably also would affect sex hormone pattern [35].

However, as for BC, a negative association between CC and intake of cereals or nonstarch polysaccharide fiber has been observed in most (but not all) epidemiological studies [review in 146, 147], the case-control studies being less convincing [see 147]. To my knowledge no prospective studies on effect of grain fiber or whole-grain products on CC incidence have been published. Recent studies suggest that the fat/fiber ratio is important also in the pathogenesis of CC because a negative association between CC and dietary fiber was found only in men with low fat consumption [148]. Epidemiological studies in Finland and Denmark point to a protective role of cereal fiber [89, 90, 129, 149], but also other factors like high consumption of fermented milk lowering colonic pH [150, 151] and supplying calcium [152, 153] [review in 154] are most likely partly responsible for the favourable CC incidence in rural Finland. Thus fermented milk may play a role for both BC (lowering effect on intestinal ß-glucuronidase) and CC risk [94, 154, 155]. As indicated above the dietary fat/fiber ratio seems to determine the degree of the enterohepatic circulation of hormonal steroids and may in this way alter the risk of hormone-dependent cancers. In experimental colon carcinogenesis this ratio determines the tumor prevalence and dietary fiber content determines the bile acid concentration and protects against the deleterious effects of fat [156, 157].

Because of the relatively high consumption of whole-grain rye bread in Finland we have been interested in studying whether different cereal products may have different effects on the CC risk factors. From these studies we have now obtained more support for the theory [21] that certain fiber-rich grain products, supplying precursors for mammalian lignan formation perhaps protecting against BC and locally having a favourable influence on intestinal bacterial composition and metabolism and mucosal cell environment, may be protective with respect to CC also by another mechanism. This is because rye bread seem to favourably influence intestinal bile acid metabolism. In a recent experiments we observed that by changing the bread consumption from a wheat fiber-free bread or from a whole-meal fiber-rich (fiber > 9 %) wheat bread to a whole grain rye bread (fiber > 8 %), significant alterations of the biochemical risk factors of CC could be obtained (see below), suggesting that the relatively small dietary change may have positively affected intestinal metabolism. The rye bread made from whole grains, not purified during milling, compared to both the fiber-free and a fiber-rich wheat bread (produced after modern milling of the grain eliminating some fractions, but containing essentially all components) increased considerably the urinary lignan excretion (R. Korpela & H. Adlercreutz, to be published). Compared to the control period no change (whole-meal) or a decrease (wheat, fiber free) was observed for the other breads. As mentioned above we have shown that lignan excretion is low in women with BC [21, 34, 57], most likely due to low intake of whole-grain bread. Furthermore, it has been shown that autohydrolyzed lignin, which is a polymer with similar basic structure as the diphenolic lignans, protects against experimental colon adenocarcinoma in rats [158]. Lignin is also known to bind deoxycholic acid very well compared to other types of fiber [159]. The effect of rye bread (200 – 300 g per day, no other cereal products consumed) on intestinal bile acid metabolism was remarkable because it considerable decreased the total free bile acid, and total and free secondary bile acid concentrations and the ratio of secondary to primary bile acids in feces (J. T. Korpela, H. Adlercreutz & R. Korpela, to be published) leaving, however, the LCA/DCA ratio unchanged. This ratio increased with consumption of the fiber-free

wheat bread. The reason for the decrease in free bile acids was a huge increase in the concentration of saponifiable (esterified) bile acids to a mean of about 46 % of total bile acids. These esters have been found to form a high proportion of the bile acids in feces in vegetarians (up to 80 %) but occur in very low amounts in CC patients (mean about 10 % of total bile acids) [160]. According to our theory the saponifiable (esterified) bile acids may not be cocarcinogenic or comutagenic as found for the aminoconjugates of these acids [16]. The reason for this may be that they are nonpolar and therefore less water-soluble which may be advantageous [152]. With the other types of bread practically no change of bile acid pattern occurred, or if any, it was in the opposite direction, particularly with respect to the fiber-free wheat bread. This is in agreement with a previous study showing no change in fecal bile acid excretion after consumption of a wholemeal bread compared to "white bread" [161]. In this connection it is of interest to note that during wartime the milling of flour resulted in much higher fiber, and possibly lignan precursor contents, which seems to have resulted in a modest decrease in colon cancer mortality [162]. These results would imply that by a simple change of the bread consumption to a daily intake of 200 – 300 g of whole-grain rye bread (or some other grain?), containing all the components of the cereal, the risk for both BC and CC could at least theoretically be reduced. Interestingly recent associations have been found both between BC [163] and CHD [164], and adenomatous polypes in colon, which are regarded as the first stage of some CC tumors.

Our results with respect to fecal bile acid metabolism are not in disagreement with the original theory of Hill *et al.* [13], but extend the theory to include the degree of "esterification" (the saponifiable bile acids have not yet been characterized). It is still most likely that the concentration of free secondary bile acids is an important factor determining the CC risk [15–20, 165, 166].

CONCLUSIONS

In conclusion, it seems that a Western diet with high fat and protein intake and low intake of fiber, complex carbohydrates and whole-grain products is associated with high plasma sex hormone levels and low SHBG, 5α-DHT, high %FT and %FE2, high urinary and low fecal excretion of estrogens, high urinary catecholestrogens excretion and 2-hydroxy-E1/4-hydroxy-E1 ratio, and low urinary excretion of lignans and isoflavonic phytoestrogens. These compounds apparently are protective with regard to cancer by many different mechanism. With respect to plasma hormones (except 5α-DHT), urinary lignans and equol we found this pattern in the postmenopausal BC women in Boston. Furthermore such a diet leads to unfavourable plasma lipid levels and intestinal bile acid metabolism most likely increasing the risk for both CHD and CC. In the study in Finland, where the BC and CC incidences are much lower than in USA, the hormonal pattern in the young BC patients was very similar to that of the control omnivorous and vegetarian women (33, 34), probably because of the relatively high intake of grain products [41] in all groups studied, but mean grain intake was still lowest in the BC group. The situation may be different in premenopausal compared to postmenopausal women, but still nothing speaks against the theory that diet is an important BC risk factor. This seems to be the fact particularly in the postmenopausal women, but probably and perhaps to a lesser degree, also in young women. All dietary components seem to have their specific role(s) in influencing sex hormone metabolism as described above and in this way a wrong diet may influence the development of BC and other sex hormone-dependent cancers in the promotional stage of the disease. More work is still needed, but already now it seems that the above-mentioned studies showing very distinct associations between diet and sex hormones and SHBG and diet and fecal bile acid pattern fit rather well with the view of the epidemiologists, that Western diet is the main factor causing the high incidence of hormone dependent cancers and CC in the Western world. Furthermore, many significant biochemical and hormonal connections between BC and other Western diseases, like CHD, exist, indicating that the same type of diet partly by the same mechanisms may be responsible for several of these diseases.

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lism and in addition with some interesting associations between the various diseases. Further support for the previously proposed extension [21] of the "fiber hypothesis" of Burkitt & Trowell [see 10] has now been obtained and will be discussed including not only BC and CC but also other Western diseases.

EFFECT OF VARIOUS MACRONUTRITIENTS ON SEX HORMONE METABOLISM

Effect of fiber

The development of a radioimmunological chromatographic method for the assay of the very low amounts of estrogens present in feces of men and nonpregnant women [22] made it possible for the first time to obtain a complete view of the effect of diet on the enterohepatic circulation of estrogens in man.

A high intake of fiber in premenopausal women increases fecal wet and dry weight, which correlates positively with all three unconjugated estrogens and total estrogens in feces [23]. In the same study also postmenopausal women were investigated (H. Adlercreutz, E. Hämäläinen, S.L. Gorbach, B.R. Goldin, J.T. Dwyer, M.N. Woods, unpublished results) and the same results were found. Furthermore, in the postmenopausal women we found positive associations between total fiber and grain fiber intake, and fecal estrone (E1) and estradiol (E2) excretion (list of abbreviations in Table I). Fat intake on the other hand seems to have a negative association with fecal excretion of estrogens [24] and therefore the dietary fat/fiber ratio of the postmenopausal women living in Boston shows highly significant negative correlation with fecal estrogen excretion (above-mentioned unpublished study). It is suggested that the dietary fat/fiber ratio determines the degree of interruption of the enterohepatric circulation of steroids, but the type of fiber plays also a significant role (see below).

In premenopausal women fecal weight and fecal estrogen excretion was found to correlate negatively with urinary estrogen excretion [23]. Particularly important was the observation of a negative correlation between fecal estriol (E3) and urinary E3-3-glucuronide (E3–3G) excretion. Urinary E3-3G is a specific metabolite of the intestinal mucosal cell and the end-product of estrogen metabolism and therefore a good indicator of the extent of the enterohepatic circulation of estrogens, particularly of E3 and other 16-hydroxylated and polar estrogens in man [25]. In a study carried out in Helsinki in premenopausal women it was found that total fiber intake and grain fiber intake/kg body weight were negatively associated with the excretion of 10 of the 13 estrogens measured in urine [26].

Fecal estrogen excretion shows a negative association with plasma E1 and E2 [23] and later on a direct negative correlation between total fiber intake and plasma E1 and E2 [24] and estrone sulfate (E1S) [27] could be observed in young women. Similar findings in men have been reported, but in addition to the negative correlation between crude fiber intake and plasma E2, higher fiber intake is associated with lower plasma testosterone (T) levels [28–30]. The reason for reduced intestinal reabsorption and increased elimination of estrogens by the fecal route in subjects consuming much fiber seems to be the larger fecal bulk and decreased concentration of intestinal β-glucuronidase [21, 23, 25]. The latter phenomenon reduces hydrolysis of the biliary steroid conjugates, an event necessary for their reabsorption. Some fibers have also the property of binding sex hormones, particularly non-polar estrogens [31, 32].

Preliminary results in the large study in Helsinki, called the "Finlandia study" revealed significant positive correlations between intake of total fiber, vegetable fiber and fiber from fruits and berries and plasma sex hormone binding globulin (SHBG) and negative associations between the intake of the same fibers and plasma % free estradiol (%FE2). Furthermore, total fiber, grain fiber and vegetable fiber intake correlated negatively with plasma % free testosterone (%FT) [33, 34]. The new results obtained in in postmenopausal Boston women [23, 24] agree well with the above-cited publications in that significant negative correlations were found between intake of total fiber, grain fiber and non-grain fiber and plasma androstenedione (A), T, FT [35] and E1. In addition intake of fruit and vegetable fiber and grain calories correlated negative-

ly with plasma E1 [estrogen results unpublished, see 27].

It may be concluded that high fiber intake is associated with low levels of sex hormones in plasma, high SHBG and low %FE2 and %FT causing a reduction in the bioavailability of the hormones, which theoretically would reduce the risk of hormone-dependent cancer. The proposed mechanisms involved in changing the SHBG level will be discussed in the sections on dietary protein, and lignans and isoflavonic phytoestrogens.

Effect of protein

Most of the studies on the effect of protein intake on hormone metabolism have been carried out by altering the protein/carbohydrate ratio of the diet. Using this technique it was found that a high dietary protein/carbohydrate ratio decreases the plasma level of SHBG and T and that a low ratio has the opposite effect [36, 37]. Furthermore a high protein diet considerably diminished 4-ene-5α-reduction of T and enhanced 2-hydroxylation of E2 [38, 39]. By measuring the estrogen profile in urine by capillary GC-MS in premenopausal women [40, 41] we could recently confirm that a high dietary protein/carbohydrate ratio results in high urinary excretion of catecholestrogens. A new finding was that the dietary protein/carbohydrate ratio is highly significantly and positively associated with the urinary 2-OH-E1/4-OH-E1 ratio. Furthermore the lowest mean ratio (= 3.6) was found in vegetarians, followed by the omnivores (= 4.3) and the highest was found in the BC patients (= 7.1) (BC *vs.* vegetarians $p < 0.005$; BC *vs.* omnivores $p < 0.02$), who had the highest dietary protein/carbohydrate ratio due to low grain intake. It may be mentioned that this ratio was recently found to be 2.0 in the same Oriental migrant women in Hawaii [42], which were previously studied by us [24].

Effect of carbohydrates

In the above section the effect of changes in the dietary protein/carbohydrate ratio was discussed. Some further information as to the possible effect of carbohydrates on sex hormone metabolism derives from studies in which dietary intake of various macro- and micronutritients were correlated with plasma and urinary hormone levels.

Recently we found that postmenopausal women living in Boston showed significant negative associations between carbohydrate intake and plasma T, E1 and E2 [35,43]. Furthermore in the same study the intake of grain calories showed negative correlations with plasma A, T, DHEAS, and E1. The intake of carbohydrates also showed a weak but significant positive correlation with fecal E1 excretion (estrogen results unpublished).

In the corresponding Finnish study in 33 premenopausal women [40–42], studied twice during a year, we found some other interesting correlations between carbohydrates and sex hormones. Urinary 2-OH-E1/4-OH-E1 ratio correlated positively with protein/carbohydrate ratio of the diet and negatively with carbohydrate, starch, total fiber and grain fiber intake. Urinary 4-hydroxyestrone excretion correlated positively with total and grain fiber intake and plasma SHBG and negatively with %FE2 and %FT. Starch intake was negatively associated with urinary E3-3-glucuronide, the specific marker of the enterohepatic circulation of estrogens, suggesting partial interruption of this circulation in subjects with high starch intake. Carbohydrate intake was negatively associated with plasma E1S, the mean level of which was highest in the BC group. Plasma DHEAS on the other hand was strongly positively associated with plasma E1S, and less strongly with %FE2 and negatively associated with urinary 16-hydroxylated estrogens and enterolactone (Enl) [27]. Enl mainly derives from precursors in grain and its urinary excretion reflects both the intake of fiber in general [44] and whole-grain products in particular. The results indicate that it is difficult to separate the effect on hormone metabolism of complex carbohydrates from that of fiber.

Effect of fat

Oriental women living in East Asia and at low risk for BC consume a very low-fat diet (usually < 20 % of calories). Studies on the urinary excretion of E1, E2 and E3 have shown that they excrete lower amounts of E1 and E2 and similar amounts of E3

compared to women in Western countries [24, 45, 46]. In other studies in vegetarians living in Western societies the picture has not been so clear, but there has been a trend towards lower urinary E1 and E2 values and similar or slightly higher E3 values in the vegetarians [47, 48]. Thus a vegetarian or semivegetarian diet seems to be associated with relatively high E3 formation. The simultaneously higher fecal excretion of E3, however, reduces urinary E3 levels leading to varying quantitative results for E3 in urine, depending mainly on the nature of the fiber in the food and the quantity of both dietary fiber and fat. Simultaneously there seems to be a reduction in the relative concentration of 2-hydroxyestrogens, particularly in Oriental women and a relative increase in 4-hydroxylation [41, 42], which means that the main metabolic pathways in these women unexpectedly seems to lead to biologically more active estrogens. However, it must be remembered that their plasma and urinary E1 and E2 levels were shown to be low [24] and the net biological estrogen effect may in any case be less. It has also been shown that the luteal phase E2 values are lower in young women following a low-fat diet for 2 months [49].

Women living in Africa consuming low-fat habitual diets [50] and Oriental migrants in Hawaii [24] have low plasma androgen levels compared with women on a Western diet. These observations are in agreement with the results obtained in postmenopausal omnivorous and vegetarian women and postmenopausal women with BC showing the lowest plasma A, T, %FT, %FE2 and DHEAS and highest SHBG (after correction for weight) in the vegetarian women, who had the lowest dietary fat/fiber ratio of the three groups [35, 43] The lower DHEAS in vegetarians is in agreement with recent results showing that plasma E1S levels are lower in women on a low-fat high-fiber diet compared to a typical Western diet [51] because the levels of these sulfates show a significant association [27 and unpublished results]. In correlation analysis a Western-type diet was found to be associated with the hormonal pattern observed in the postmenopausal women with BC, but this was obviously not entirely due to the diet [35].

It seems justifiable to conclude that a high protein and fat and low grain, complex carbohydrates and fiber intake leads to higher plasma levels of biologically active sex hormones and lower SHBG, with a clear tendency to lower 16α– and 16β-hydroxylation [42] and higher 2-hydroxylation of estrogens and higher urinary 2-hydroxy-E1/4-hydroxy-E1 ratio. The possible role of these alterations of hormone levels as etiological factors in hormone-dependent cancer will be discussed below. It should be mentioned that opposite results with regard to 16α-hydroxylation of estrogens and fat intake have been published [52, 53], and these results will be discussed in the section on BC.

LIGNANS, ISOFLAVONES, AND SEX HORMONE METABOLISM

Since the detection and identification of mammalian and later also of plant lignans and isoflavonic phytoestrogens in the human organism, many studies on their biological role in health and disease have been carried out. Several reviews [33, 54–56] on the topic have recently been published. These diphenolic compounds occurring in high amounts in the organism have numerous different biological activities of which most seem to make them candidates for a role as protective substances with regard to cancer and particularly hormone-dependent cancers [12, 21, 33, 34, 54, 56–64].

To date 15 lignans and isoflavonic phytoestrogens, all diphenolic in character, have been identified in human urine and some of them also in other biological materials [54, 56, 65, 66]. Of these 7 can now be measured by combined capillary gas chromatography–mass spectrometry utilizing the selective ion monitoring technique and isotope dilution mass spectrometry using deuterated internal standards [58, 67]. The lignans enterolactone (Enl), enterodiol (End) and matairesinol (Mat) and the isoflavonic phytoestrogens daidzein (Da), equol (Eq), O-desmethylangolensin (O-Dma) and genistein (Gen) have all weak estrogenic activity, but antiestrogenic activities have also been described [reviews in 54, 56]. Many plant lignans have been shown to have anticarcinogenic, antiviral, bactericidic and antifungal activities. In collaboration with Dr Larry Vickery (Irvine, California) it was shown that Enl and a theoretical

intermediate between Mat and Enl are moderate inhibitors of placental aromatase and compete with the natural substrate androstenedione for the enzyme. Enterolactone was also able to inhibit aromatase intracellulary in cell cultures suggesting that these compounds may function as natural aromatase inhibitors. Other experiments show that these diphenols are readily transferred from cell culture media into the cells and that they may inhibit cancer cell growth, because antiproliferative effects of synthetic and naturally occurring flavonoids on tumor cells of the human breast cancer cell line, ZR-75-1, were reported [59]. Furthermore, inhibitory effects of such compounds on mitogen-induced proliferation of human peripheral blood lymphocytes were demonstrated [60].

Genistein, one isoflavonic compound identified by us in human urine is a specific inhibitor of tyrosine-specific protein kinases [61-64]. Protein-tyrosine kinase activity is associated with cellular receptors for epidermal growth factor (EGF), insulin, insulin-like growth factor I (IGF-I), platelet-derived growth factor (PDGF) and mononuclear phagocyte growth factor (CSF-1), suggesting that the enzyme plays a role for cell proliferation and transformation. The enzyme has also been associated with oncogene products of the retroviral src gene family and is correlated with the ability of retrovirus to transform cells [literature in 61–64].

In collaborative studies with Dr Jim Clark and associates we have found that several plant and mammalian lignans and isoflavones compete with E2 for the rat uterine nuclear estrogen type II binding site (unpublished results). These sites seem to constitute a component of the genome which regulates estrogen-stimulated uterine growth [68, 69]. It was found that some flavonoids like luteolin, quercetin and pelargonin inhibit E2 binding to this receptor and in this way uterine cell growth. They also inhibited growth of MCF-7 cells in culture, and *in vivo* E2 stimulation of immature rat uterus [70]. The structure of these compounds are very similar to those of the isoflavones and in fact all are diphenols. The most effective with regard to type II site binding of the diphenolic compounds found and measured by us in human urine seem to be the isoflavones daidzein and equol, but also some lignans like matairesinol, isolariciresinol and enterolactone show competition (competition observed at concentrations from 10 to 100 nmol/l). Later an endogenous inhibitor of the nuclear type II binding site was identified as being methyl p-hydroxyphenyllactate [71], which can be a metabolite of both exogenous flavonoids and tyrosine. Because this compound cannot be found in cancer tissue it was postulated that uncontrolled growth and proliferation of malignant cells is directly related not only to the permanent stimulation of nuclear type II binding sites by estrogens or other compounds, but also to very low to nonmeasurable levels of the competitive inhibitor methyl p-hydroxyphenyllactate [71]. In our opinion it seems that probably many of these phenolic compounds may have a synergistic action as it is unlikely, because of close structural similarities, that only one of them inhibits cell growth. The compound found by Markarevich et al. [71] was isolated from fetal bovine serum, probably a very rich source of many flavonoids and phytoestrogens and their metabolites. The concentration of the new monophenolic compound in biological fluids and tissues in human subjects has to my knowledge not been measured. The possible growth-inhibiting and antiproliferative role of individual flavonoids and their metabolites with regard to hormone dependent cancer is a new interesting area of research that needs much further studies.

Of the isoflavones the strongest estrogens are Eq and Gen, but they are still very weak estrogens compared to E2 and E1. It is unlikely that all their other biological effects are related to their estrogenicity. Quantitative results indicate that lignans and isoflavonic phytoestrogens are normal constituents of human urine and are excreted in large amounts particularly by vegetarians (both lignans and phytoestrogens) [33, 34, 58], by subjects consuming large amounts of whole-grain products, vegetables and berries, which all are associated with high lignan excretion [33], and by the Japanese consuming traditional Japanese diet (mainly isoflavonic phytoestrogens, due to intake of soy products) [33, 72]. In omnivorous Finnish subjects the excretion of Gen, the specific inhibitor of protein tyrosine kinase, was found to be between

10 and 1,500 nmol/24 h (usually 1-4 times that of Da). When investigating a few Japanese subjects consuming a traditional diet the excretion was very high ranging from 1,250 to 15,500 nmol/24 h (!) (in collaboration with H. Honjo and coworkers), about 1.5 - 3 times higher than that of Da. As mentioned Da shows antiproliferative activity with regard to BC cells [59]. Particularly low excretion of these compounds has been observed in BC patients and in subjects consuming a low-fiber diet, especially a diet low in whole-grain products and beans [23, 24, 49, 64, and unpublished results]. Particularly low excretion has been observed in BC patients and in subjects consuming a low-fiber diet, particularly a diet low in whole-grain products [33, 34, 58, 73].

It has now been demonstrated that the mammalian lignans Enl and End are formed from precursors, such as the plant lignans matairesinol and secoisolariciresinol, which are consumed and then structurally modified by intestinal bacteria [56]. Eq and O-Dma are most likely formed by intestinal bacterial action from formononetin (For) and Da present in food stuffs like soy products [72, 74]. However, these compounds are also present in cow milk [75] formed from *e.g.* For in clover by intestinal bacteria in the gastrointestinal tract of the cow [55], and may therefore be consumed by human subjects as such. Because of the close association of lignan excretion with fiber intake [21, 33, 44] it is likely that the plant lignans are localized close to the outer fiber-containing layers of the grain containing phytin, polyphenols, enzyme inhibitors and other compounds usually regarded as antinutritional factors [76].

Recently, we suggested that the lignans and isoflavonic phytoestrogens, which all are diphenols, perhaps together with other similar compounds, stimulate SHBG synthesis in the liver and in this way reduce the biological effects of sex hormones [27, 33, 34]. An increase in SHBG results in lowering of %FT and %FE2 and reduction of both the albumin-bound and the free fraction of the sex hormones. This reduces the metabolic clearance rate (MCR) of the steroids and reduces in this way their biological activity.

In Finnish women total fiber intake, total fiber intake/kg body weight and grain fiber intake/kg body weight correlate positively and dietary fat/fiber ratio negatively with urinary excretion of total lignans and isoflavonic phytoestrogens [33, 34]. The excretion of the two diphenolic groups of compounds and also Enl alone in both pre- and postmenopausal Finnish women correlate positively with plasma SHBG and negatively with plasma %FE2 and %FT [33, 34 and unpublished results]. It is well known that oral estrogens, in contrast to parenterally administered ones, markedly stimulate SHBG synthesis [77, 78] and we therefore suggest that these positive associations between urinary lignan and phytoestrogen excretion and SHBG is due to stimulation of SHBG synthesis by these week estrogens entering the portal circulation in very high amounts. This also would explain the higher SHBG values seen in vegetarians [79] including such vegetarians whose diet does not contain low amounts of proteins [34]. High protein diet has been found to lower plasma SHBG [36, 37].

Furthermore urinary Enl excretion in these Finnish women correlates negatively with plasma DHEAS and luteinizing hormone (LH) (unpublished observations). The latter observation has to be evaluated in detail, but it is possible that the effect on sex hormone metabolism of these weakly estrogenic compounds may also be mediated via an effect on the hypothalamic–hypophyseal endocrine system. Plasma DHEAS is low in vegetarians and is negatively associated with the dietary intake of unsaturated fatty acids [35].

DIET, SEX HORMONES AND BREAST CANCER

In an extensive review about 10 years ago Dao concluded that studies of estrogen metabolism in BC has provided only controversial results and that they are inconclusive at best [80]. The results described above indicate clearly that studies on sex hormone metabolism in cancer cannot be carried out without careful dietary evaluation in the subjects studied. It is therefore not surprising that no consensus as to the association between sex hormone changes and BC has been reached, because very few studies include both detailed dietary records and hormonal investigations.

E11

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Variations in the Content of Plant Oestrogens in Red Clover-Timothy-Grass during the Growing Season

By *K. Kallela, I. Saastamoinen* and *E. Huokuna*

Kallela, K., I. Saastamoinen and E. Huokuna: Variations in the content of plant oestrogens in red clover-timothy-grass during the growing season. Acta vet. scand. 1987, 28, 255–262. – The investigation concerned variations in the plant oestrogen content of red clover-timothy swards receiving the same basic fertilization, but with different plant compositions and levels of nitrogen fertilizing.

The oestrogen content of pure red clover was high in the early spring and declining by midsummer. The oestrogen content in aftermath remained high and compared to the age of the growth was greater than that of the spring crop. The age of the aftermath from the preceeding harvest, as well as the time of harvesting affected the content.

The plant oestrogen content of red clover grown in the mixed swards was on average somewhat higher that from a pure red clover sward. The plant oestrogen content of a pure timothy sward was usually negligible.

Nitrogen fertilization diminished the share of red clover in the mixed swards and thus the plant oestrogen contents of those swards as well. The effect was very notable in the spring crop. The average content of aftermath was consistently higher than that of the spring crop.

Nitrogen fertilizer had a loowering effect on the estrogen content of pure red clover as well as on the percentage of crude protein in clover. The study found a strong correlation between the plant oestrogen and crude protein contents of red clover. Apparently both are due much to the same factors despite their divergent chemical compositions.

Changes occurring in the total plant oestrogen content are determined predominantly by alternations in the contents of formononetin and biochanin-A, which were usually parallel. Compared to these changes, the quantities and respective variations of daidzein and genistein were insignificant.

plant composition; nitrogen fertilization.

Introduction

In plants the quantity of oestrogens chiefly depends upon the plant species and variety but is also variable in the same plant during the growing season. It is generally high in the spring during the luxuriant growth period, however ample amounts of plant oestrogens have also been found in autumn aftermath (*Kallela* 1964). The formation of oestrogens is probably influenced by many factors such as for example climate, fertilization and soil. In Finland, the most commonly cultivated fodder plants are timothy and red clover. Oestrogenic activity in red

clover is comparatively great, whereas timothy contains negligible amounts of plant oestrogens (*Kallela* 1974). Exactly how the quantities of plant oestrogens vary in plants during the course of the growing season has not yet been systematically shown. Therefore, it was considered well founded to investigate the variations during one growing season for the content of plant oestrogen in red clover-timothy swards with varying plant compositions and different levels of nitrogen fertilizing.

Materials and methods

The red clover-timothy swards studied were cultivated during the summer of 1984 at the South Savo Agricultural Research Station at Mikkeli (61°, 40'). The variety of red clover used was the Finnish tetraploid Tepa and the timothy variety the Finnish Tammisto.

The plant compositions to the swards were as follows:

1. pure red clover
2. clover-rich red clover-timothy; 14 kg red clover seed and 6 kg timothy seed per hectare were used for seeding the sward
3. clover-poor red clover-timothy; 6 kg clover and 14 kg timothy per hectare were used
4. pure timothy.

The basic fertilization was identical for all swards; for both harvests 400 kg/ha trace element enriched PK-fertilizer was applied (2-8-12). The timothy and mixed swards in addition received treatments of 0, 50 and 100 kg/ha nitrogen fertilizer for both the spring and aftermath harvests. Plant growth commenced on May 1. Yields were gathered weekly. The first moving of spring growths took place on May 29 when the plants were 4-weeks old and the last mowing was on July 10 when their corresponding age was 10 weeks.

Aftermath yields were gathered on July 31, and on August 7, 14 and 21, respectively. At the time of the first mowing, 8 and 9 weeks had elapsed from the spring mowings (previous mowing on May 29 and June 5), at the second, 7 and 8 weeks (June 12 and 19), at the third, 6 and 7 weeks (June 26 and July 3); and the last moving on August 21 took place 6 weeks from the previous mowing on July 10, 1984.

Immediately after mowing, representative samples for plant oestrogen determinations were collected from the swards. The samples were finely ground in a meat grinder, allowed to stand for 1/2 h at + 37°C in order for the plant oestrogens to hydrolyze (*Francis & Millington* 1965), then mixed into an ample amount of absolute alcohol and stored until analysis.

For the high performance liquid chromatographic determination the samples were filtered. From the filtrates the oestrogenic isoflavones formononetin, biochanin-A, genistein and daidzein were determined by an earlier described method (*Kallela & Saastamoinen* 1978) which was modified so that the conditions were as follows:

Equipment:	Perkin-Elmer 1220 liquid chromatograph UV/detector LC-55 Perkin-Elmer at 254 nm Sigma 10 lab data system
Pre column:	3 µm 3 cm C-18 258-0160
Column:	5 µm 10 cm HS-5 HCODS 258-0152 P-E
Eluent:	40 % acetonitrile in water
Flow rate:	1 ml/min
Temperature:	55°C
Chart speed:	5 mm/min
Pressure:	1500 PSI
Sample size:	2 µl

The fluorescent isoflavones (daidzein and formononetin) were also determined by a

LS-4 fluorometric detector. Commercial plant oestrogen preparations (K & K Laboratories, Inc.) served as standards.

Due to practical difficulties, coumestrol, the content of which is known to be negligible in the plants studied, if present at all, was not determined (*Kallela* 1964).

Results

The results are presented graphically in Figs. 1–5. The average plant oestrogen contents of pure red clover from all swards containing red clover is presented in Fig. 1. Thus the figure includes the pure red clover swards, the selected clover from the clover-rich red clover-timothy swards (fertilizations of 0, 50 and 100 kg N/ha), and the selected red clover from the clover-poor red clover-timothy swards (fertilizations of 0, 50 and 100 kg N/ha).

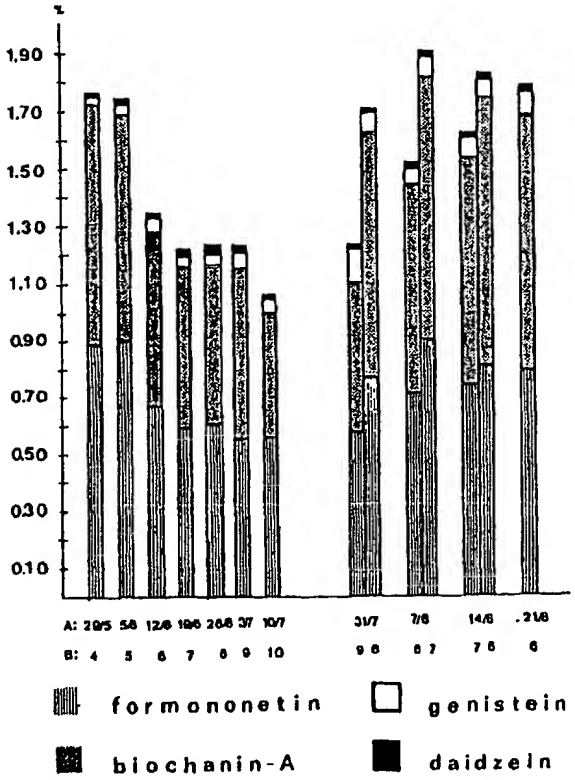

Figure 1. Average plant oestrogen content (% of DM) of all red clover samples.
A = mowing date. B = crop age (in weeks).

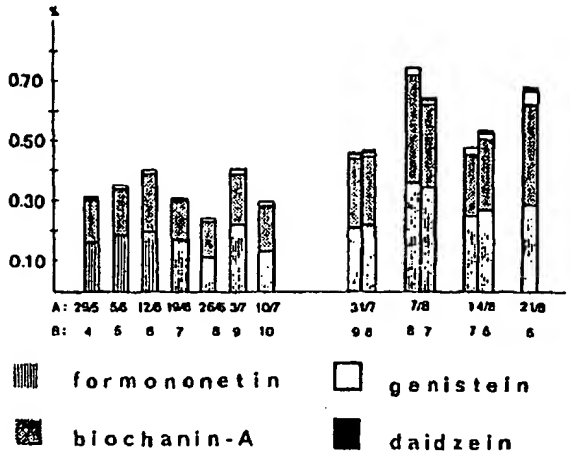

Figure 2. Average plant oestrogen content (% of DM) of cloverrich swards.
A = mowing date. B = crop age (in weeks).

Fig. 2 illustrates the contents of the variously fertilized (0, 50 and 100 kg N/ha) clover-rich, Fig. 3 those of the clover-poor and Fig. 4 shows the average plant oestrogen content of the pure timothy swards. The average crude protein and plant oestrogen

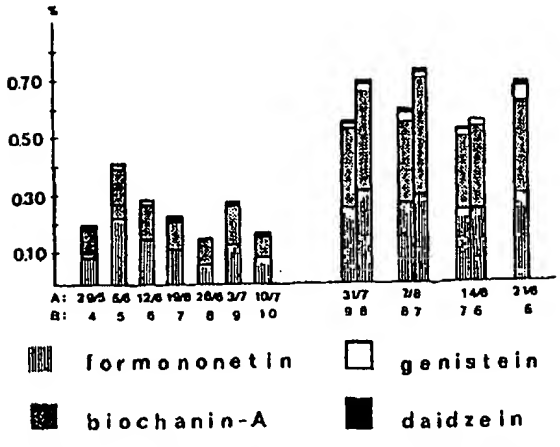

Figure 3. Average plant oestrogen content (% of DM) of cloverpoor swards.
A = mowing date. B = crop age (in weeks).

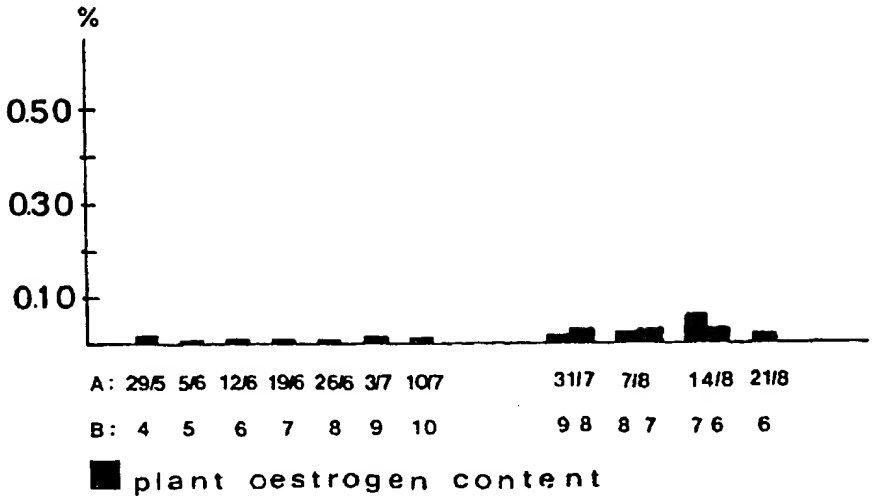

Figure 4. Average plant oestrogen content (% of DM) of pure timothy swards.
A = mowing date. B = crop age (in weeks).

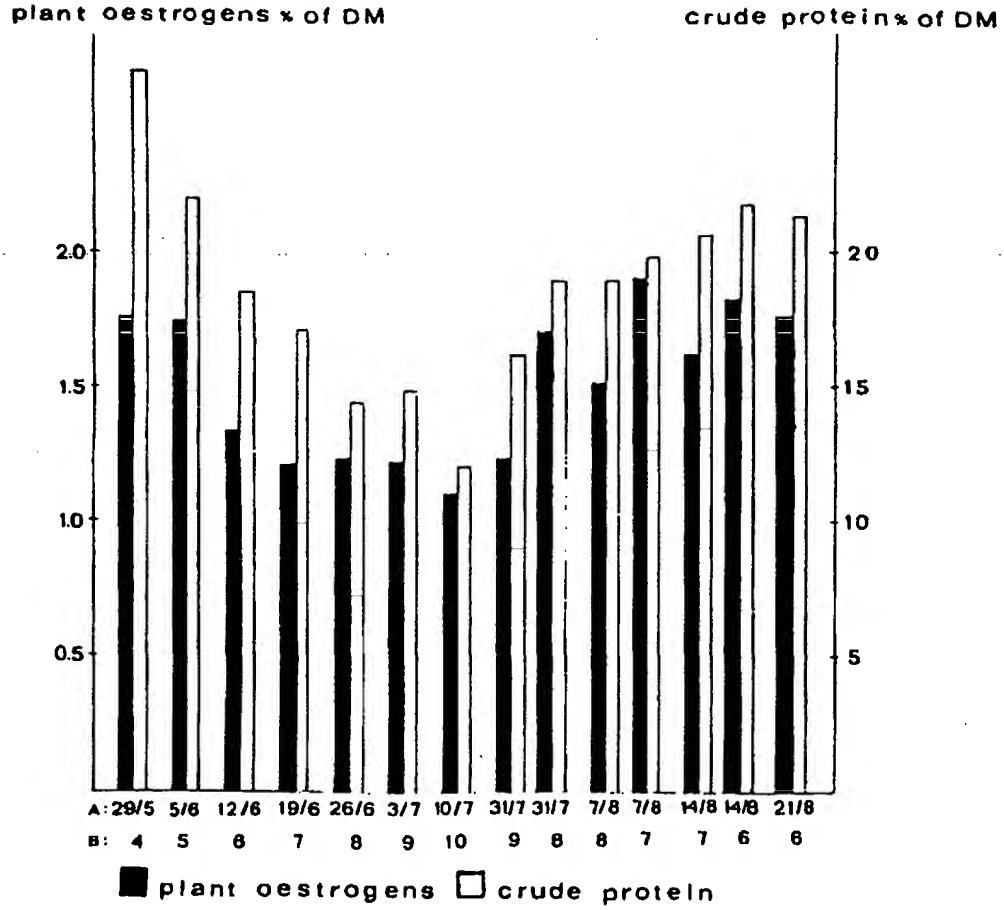

Figure 5. Average plant oestrogen and crude protein contents of all red clover samples.
A = mowing date. B = crop age (in weeks).

contents of red clover for all of the swards are presented in Fig. 5.

Detailed information concerning the plant oestrogen contents of all samples investigated can be obtained from the authors. From these data the plant oestrogen contents of red clover at different times of harvesting in pure red clover and in differently fertilized clover-rich and clover-poor swards and, correspondingly, in ungraded clover-rich and clover-poor and pure timothy swards are evident. Detailed information can also be obtained on the crude protein and dry matter contents in the red clover samples, and the harvest yields of the red clover and timothy swards.

A statistical evaluation of the results is presented in Table 1.

Table 1. Statistical relationship (regression coefficients) of plant oestrogen and crude protein content in red clover and their relationship to red clover and total harvest yields of the swards.

	Plant oestrogen content of red clover (% of DM)						Crude protein content of red clover (% of DM)					
	crude protein content of red clover (% of DM)		red clover yield (kg DM/ha)		total crop yield (kg DM/ha)		red clover yield (kg DM/ha)		total crop yield (kg DM/ha)			
	spring yield	after-math	spring yield	after-math	spring yield	after-math	spring yield	after-math	spring yield	after-math		
Red clover sward	+0.936	+0.865	−0.931	−0.929	−0.931	−0.929	−0.967	−0.776	−0.967	−0.776		
Clover-rich sward	+0.720	+0.737	−0.735	−0.791	−0.729	−0.748	−0.854	−0.699	−0.993	−0.882		
Clover-poor sward	+0.824	+0.389	−0.889	−0.816	−0.729	−0.698	−0.819	−0.648	−0.905	−0.861		

Discussion

The purpose of the investigation was to clarify the variations during one growing season in the plant oestrogen contents of Finnish red clover-timothy-grass whose N-fertilization rate and plant composition varied. The plant oestrogen content was determined in both the swards as such, and in the selected red clover of the mixed swards.

Average plant oestrogen content variation in test swards

The plant oestrogen content of the red clower swards as well as that of the selected red clover from the mixed swards of the spring yield was highest in the youngest crops, aged 4–5 weeks and declined greatly by midsummer. The plant oestrogen content rose again in aftermath and compared to the age of the crop, it was regularly higher than that of the early summer yield.

It seems apparent that especially in aftermath the plant oestrogen content is determined not only by the crop's age but also by the time period when mowing is carried out,

as aftermath of the same age mown earlier in autumn contains a greater abundance of plant oestrogens than does that harvested at a later date.

In addition to the physiological age of the plant and harvest time, the plant oestrogen content of red clover may be influenced by other growing conditions such as rainfall, temperature, etc. One Swedish study found markedly high plant oestrogen contents in plants after very cold nights in autumn (*Pettersson et al.* 1984). Likewise, it has been found that plant diseases tend to increase plant oestrogen contents (*Shutt* 1976).

Determinations performed on both the clover-rich and clover-poor swards show the plant oestrogen contents of the clover-rich swards to be greater in the spring than those of the clover-poor swards. In the spring (May 1, 1984) clover sprouts were also more abundant in the clover-rich swards (mean 53 plants/m^2) than in the clover-poor swards (30 plants/m^2). In both cases the quantity of oestrogens in aftermath clearly rose; relatively more so in the clover-poor swards. Corresponding variations could also be found in the clover yields.

In the samples from the red clover and mixed swards investigated, the total plant oestrogen content was determined mainly by the dominating quantities of formononetin and biochanin-A present which, in addition, as a rule varied in the same direction. By comparison, the contents of genistein and daidzein were rather low. They remained at the same levels throughout the entire growing season and particularly with regard to genistein, appeared to rise somewhat toward midsummer and late autumn.

The plant oestrogen content of timothy was very low, as expected. In the spring yield it was caused mainly by formononetin, and was in aftermath especially due to genistein which increased toward late autumn in the swards not fertilized with nitrogen. Because of exceptionally high genistein contents, additional thin-layer chromatographic determinations on genistein as well as on other isoflavones were performed. These investigations proved that with respect to genistein the results were apparently too high, as genistein is not completely separated from impurities by the HPLC-determination applied. However, the possible drawback of the analytical method employed on the clover and mixed sward samples is rather slight.

The plant oestrogen content of pure red clover as compared to red clover grown in mixed swards.

The growth circumstances of the test swards (soil, basic fertilization, climate) were very much similar except for the N-fertilization. By comparing the plant oestrogen content of the red clover grown in a red clover sward to that of red clover grown in unfertilized mixed swards, it is possible to draw conclusions as to the effect of plant composition in the swards on the oestrogenity of red clover.

It appears that red clover growing in unfertilized mixed swards on the average develops more plant oestrogens than does clover in pure red clover swards. The difference in aftermath is also statistically significant. A possible explanation could be the vigorous growth and great vitality of red clover which increase in autumn due to advantageous growth conditions, especially when competing with timothy for living space. This is also reflected in increased relative crop shares of red clover.

The effect of nitrogen fertilizer on plant oestrogen content

Nitrogen fertilizer had a strong effect on the clover yields and plant oestrogen contents of the mixed swards. Nitrogen fertilizer is known to favour the growth of timothy at

the expense of red clover, which clearly became evident in the present investigation also, especially in the yields of the spring crops. In the early summer yield of the mixed swards, a higher level of fertilizing decreased the summer yield of red clover in the mixed swards as well as the plant oestrogen content. The subsequent effect of nitrogenous fertilization on mown aftermath was not as obvious. Nitrogen fertilization also decreased the plant oestrogen content of pure red clover. In unfertilized red clover the average plant oestrogen content (1.562 % ± 0.330 %) is notably higher than in red clover (1.352 % ± 0.275 %) treated with 100 kg N/ha, which for its part, is markedly lower than the plant oestrogen content of red clover fertilized with 50 kg N/ha (1.537 % ± 0.372 %). In addition, when separately examining the plant oestrogen contents of the spring and aftermath crops, it can be proved that the plant oestrogen content of red clover regularly decreased with an increasing rate of nitrogen fertilizer. At the rate of 100 kg N/ha this difference is statistically significant in all groups, with the exception of the clover-poor one.

The plant oestrogen and crude protein content of red clover and harvest yields

In addition to plant oestrogen content, the present investigation examined the crude protein content of red clover and harvest yields of the swards. These investigations resulted in the observation that the changes with regard to the plant oestrogen and crude protein content of red clover are generally in the same direction. Instead, a negative correlation was discovered between the plant oestrogen content of red clover and the red clover and total harvest yield, and between crude protein in red clover and corresponding harvest yields as well.

Positive and negative correlations usually were more complete in spring swards compared to aftermath, especially concerning plant oestrogens and crude protein in heavy fertilized swards. It is possible in these cases that in autumn there are more factors effecting the oestrogen content but not necessarily the crude protein content. In an earlier published study it has been shown that among others, the plant oestrogen content can alter subsequent to cold nights (*Petterson et al.* 1984), by the effect of plant diseases (*Shutt* 1976), and also as a consequence of storage (*Ludewig* 1973, *Kallela* 1980). Such factors, however, hardly have the same effects on the crude protein content of red clover.

On the basis of this investigation, it seems apparent that the variations in the plant oestrogen content of red clover parallel with those of the crude protein content and probably owing to the same reasons, despite that chemical difference of these substances.

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Sammanfattning

Växlingar i växtöstrogenhalten i rödklöver-timotejvall under vegetationsperioden.

Undersökningen utreder förändringarna i östrogenhalten i rödklöver-timotej-valler med samma grundgödsling men med olika kvävenivå och växtkomposition.

Östrogenhalten i rödklövern från ren klövervall och vall med timotej-inblandning var hög på våren och minskade framemot högsommaren. Östrogenhalten i rödklöver från efterslåttern låg konstant på hög nivå, högre än i vårväxten. Återväxtens ålder räknad från föregående avmejning påverkade mängden av östrogen, liksom också tidpunkten för slåttern.

Rödklöver från blandvall innehöll i genomsnitt något mera östrogen än rödklöver från ren klövervall. Östrogenhalten i ren timotejvall var generellt mycket låg.

Kvävegödsling minskade rödklöverns andel i blandvall och sålunda också vallens totala östrogenhalt. Denna effekt var speciellt betydande i vårväxten. I efterslåttern från blandvall var östrogenhalten regelbundet högre än i vårväxten.

Kvävegödsling sänkte östrogenhalten i ren rödklöver och också råproteinhalten minskade. En stark korrelation förelåg mellan växtöstrogenhalten och halten av råprotein i rödklöver. Uppenbarligen är båda beroende av samma faktorer trots olikheten i kemisk komposition.

Förändringarna i östrogenhalten bestäms i huvudsak av formononetin och biochanin-A, vilkas växlingar går i samma riktning. I jämförelse med dem var daidzein- och genisteinmängderna små, liksom också växlingarna i deras koncentration.

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Sexual behaviour of ewes with clover disease treated repeatedly with oestradiol benzoate or testosterone propionate after ovariectomy

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Summary. Ovariectomized ewes, 14 with permanent clover disease infertility (affected ewes) and 14 controls, were injected daily with 40 μg oestradiol benzoate for 12 days, and run with 2 rams fitted with marking crayons. The control ewes were mated sooner ($P < 0{\cdot}05$) but both groups became refractory at a similar rate. In a second experiment, 20 similar affected ewes and 19 controls were injected daily with 5 mg testosterone propionate for 31 days and observed daily for 50 min with rams. Affected ewes again were slower to show female behaviour ($P < 0{\cdot}05$) but faster ($P < 0{\cdot}05$) to show aggression against the rams and other ewes. Over the 31 days, the incidence of female sexual behaviour declined at a similar rate in affected and control ewes. When examined in individual pen tests with oestrous ewes on Day 28, affected ewes showed more male-like courting behaviour than did controls ($P < 0{\cdot}05$). The changes in behaviour are too slight to account for the infertility but they do support the hypothesis that phyto-oestrogens can act on the ewe by some of the pathways of sexual differentiation, even after puberty.

Introduction

Ewes which have grazed oestrogenic clover pastures for several years can develop permanent infertility (Schinckel, 1948) as part of the syndrome called clover disease. Such ewes are infertile because of impaired transport of spermatozoa through the cervix (Lightfoot, Croker & Neil, 1967). This cervical dysfunction is associated with a low spinnbarkeit of the cervical mucus resulting from an abnormal responsiveness to endogenous oestrogen (Adams, 1979) and is accompanied by metaplasia of the cervix so that histologically it resembles the uterus (Adams, 1976). Oestrogen has not been reported to cause this type of histological change in adult females of other species, although analogous changes may be caused by oestrogen during the period of organogenesis (Forsberg, 1969).

Permanently affected ewes exhibit slightly less female sexual behaviour than do controls after a single dose of oestradiol benzoate (Adams, 1978). The present study was carried out to see whether the behavioural responses of affected ewes to repeated treatment with oestradiol benzoate or testosterone propionate were altered. Some of these results have been referred to previously (Adams, 1981).

Materials and Methods

Two studies were carried out on 8-year-old Merino ewes which had been ovariectomized for at least 1 year. Half of the ewes had grazed a highly oestrogenic pasture of Yarloop subterranean clover for 3 years and only 11% had lambed after the last year of exposure. The control ewes originated from

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the same group, but had grazed non-oestrogenic pasture during this period and were of normal fertility (76% lambing). Subsequently all the ewes were run together on non-oestrogenic pasture for 4 years.

The effects of 40 μg oestradiol benzoate injected i.m. in oil were studied in 14 affected ewes and 14 controls which were injected daily for 12 days. The ewes were run with 2 vasectomized rams fitted with a harness and marking crayon. Each day the colour of the crayon was changed and the ewes marked were recorded.

The effects of daily i.m. injection for 31 days with 5 mg testosterone propionate in oil were studied in other ewes from the same flock (19 control and 20 affected ewes). The ewes were individually identified with a large number painted on the side, and were run in 2 large pens. Each day, 2 active rams were run in each pen for 50 min and the sheep were observed from seclusion. The number of times each ewe showed female sexual behaviour (standing to be mounted, soliciting the ram, looking over the shoulder at the ram and tail fanning; Banks, 1964) was recorded. The frequency was also recorded for bunting of rams and other ewes, and rubbing the side of the face or the horn buds on pen divisions or on other ewes in a manner similar to the rubbing and bush-threshing behaviour described by Grubb & Jewell (1973). The relative incidence of masculine sexual behaviour (ano-genital sniffing, Flehmen reaction, and courting by pawing or nudging; Banks, 1964) was determined by observing the ewes individually in a pen with 3 other oestrous ewes for 7 min on Days 14, 21 and 28 of treatment.

Data were analysed statistically by the t test or, if the variances were not homogeneous, by Wilcoxon's two-sample rank test.

Results

Oestradiol benzoate

All of the ewes displayed oestrus, but the onset of mating was earlier and less variable in the control than in the affected ewes (2·5 ± 0·3 compared with 4·0 ± 2·5 days, $P < 0.05$ Wilcoxon's test). The control and affected ewes continued to be marked by the ram for a similar time (5·1 ± 0·8 and 4·6 ± 0·8 days, respectively) and the mean for the last day on which matings occurred was not significantly different for the two groups (8·1 ± 0·7 and 8·8 ± 0·7 days).

Testosterone propionate

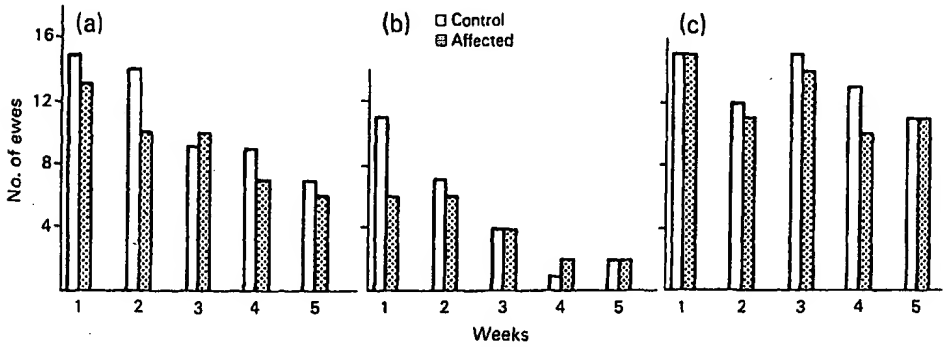
As shown in Table 1, the affected ewes treated with testosterone propionate took longer to stand and be mated by the ram ($P < 0.05$, Wilcoxon's test) and showed less soliciting behaviour in the first week of study (Table 1; $P < 0.05$). The number of ewes showing soliciting behaviour and the

Table 1. Female sexual behaviour in a group of 19 clover-affected and 20 control ovariectomized ewes during the first 7 days of daily treatment with 5 mg testosterone propionate

	No. of days to first observation		No. of times behaviour observed	
	Control	Affected	Control	Affected
Ewe accepted mounting by ram	1·73 ± 0·15 (16)	3·14 ± 0·51* (15)	3·47 ± 0·56	2·65 ± 0·58
Ewe solicited ram	4·9 ± 1·1 (14)	7·3 ± 1·3 (11)	2·68 ± 0·78	0·85 ± 0·42*
Ewe looked over shoulder at ram	3·9 ± 1·1 (15)	5·3 ± 1·7 (15)	5·2 ± 1·2	4·1 ± 0·9
Tail fanning	5·0 ± 1·2 (12)	6·6 ± 2·3 (7)	1·2 ± 0·3	0·9 ± 0·5

Values are mean ± s.e.m. Figures in parentheses indicate the number of ewes showing this behaviour.
* Value significantly different from control, $P < 0.05$.

number which stood to be mounted by the ram declined over the 5-week treatment period (Text-fig. 1). The affected and control ewes did not differ from one another in the rate of decline in responses with continued treatment.



Text-fig. 1. Total numbers out of 19 control or 20 affected ewes which (a) accepted mounting, (b) solicited, and (c) looked over their shoulder, in each week, during daily 50-min observation periods.

Some ewes challenged and head-bunted the rams, and sometimes other ewes as well. Such aggressive behaviour was observed earlier ($P < 0.05$) in the affected (mean onset 15·7 ± 1·6 days) than in the control (21·8 ± 2·1 days) ewes, and did not appear to be related to female sexual behaviour. Ewes also rubbed the poll or the side of the face on pen divisions or on other ewes; this behaviour was also exhibited sooner by affected ewes than by controls (6·0 ± 1·0 compared with 10·8 ± 1·4 days, $P < 0.05$).

When ewes were tested for male sexual behaviour, the total number of ewes showing courting behaviour increased after the first test (Table 2; 12 on Day 14 compared with 21; $\chi^2_1 = 4.25$, $P < 0.05$). In each test, affected ewes showed more courting behaviour than did controls and, by Day 28, this difference was statistically significant (Wilcoxon's test, $P < 0.05$; Table 2). There was no significant difference between groups or tests in Flehmen behaviour or in ano-genital sniffing.

Only 2 ewes (both controls) were observed to mount other ewes throughout the study.

Table 2. Number of times that 19 control and 20 clover-affected ovariectomized ewes injected daily with 5 mg testosterone propionate showed male behaviour during pen tests

	Day 14		Day 21		Day 28	
	Control	Affected	Control	Affected	Control	Affected
Courting	8·8 ± 5·1 (6)	11·7 ± 4·3 (6)	7·7 ± 4·1 (9)	10·8 ± 3·0 (12)	6·4 ± 4·5 (7)	14·0 ± 2·8* (11)
Flehmen	2·5 ± 0·8 (6)	1·6 ± 0·4 (5)	2·3 ± 1·3 (4)	2·9 ± 1·1 (8)	1·2 ± 0·2 (6)	1·4 ± 0·2 (5)
Ano-genital sniffing	3·5 ± 0·5 (13)	4·1 ± 0·9 (11)	3·7 ± 0·8 (14)	3·6 ± 0·6 (16)	3·6 ± 0·7 (14)	4·5 ± 1·6 (12)

Values are mean ± s.e.m. for the no. (in parentheses) of ewes showing the behaviour.
* Significantly different from control, $P < 0.05$.

Discussion

The behaviour of the ewes in the present study was consistent with that observed previously in ewes treated with oestradiol benzoate (Adams, 1978) or testosterone propionate (Signoret, 1975). Adams (1978) showed that mating and soliciting behaviours in oestradiol-treated ewes were more dose-

dependent than was tail fanning or looking over the shoulder, and in the present study the former behavioural features were also the most sensitive indicators of a difference between affected and control ewes treated with testosterone propionate. In the previous study on ovariectomized ewes (Adams, 1978), delayed onset or reduced incidence of female sexual behaviour was observed in clover-affected ewes given a single injection of oestradiol benzoate after treatment with progesterone for 10 days. The similar delay in onset of female mating behaviour in affected ewes given oestradiol benzoate alone in the present study indicates that the impairment results from an altered responsiveness to the oestradiol, and not from a reduced effectiveness of progesterone priming.

It is unlikely that the relatively minor changes in sexual behaviour in affected ewes have any direct bearing on the infertility. However, the nature of the changes does provide an insight into other changes which have been observed in permanently affected ewes. The uterine-like histological metaplasia of the cervix in affected ewes is best interpreted as an oestrogen-dependent differentiation (Lightfoot & Adams, 1979). In addition, ovariectomized affected ewes have increased protein and glycoprotein synthesis in the uterus and cervix and increased epithelial cell keratinization in the vagina, in the absence of any hormonal stimulation (Tang & Adams, 1981). Similar changes have been reported in female rodents treated neonatally (i.e. at the time of sexual differentiation) with oestrogen or testosterone (Kohrman & Greenberg, 1968; Takasugi & Kamishima, 1973). Such animals also have decreased female sexual behaviour and increased male behaviour (Phoenix, Goy, Gerall & Young, 1959; Gorski, 1973; Whalen & Etgen, 1978) and increased aggressive behaviour (Bronson & Desjardins, 1970) when stimulated with hormonal steroids during adult life. The parallel between the results of the present study and the organizational effects of neonatal oestrogen or testosterone on sexual behaviour in rodents is obvious. A similar parallel between the organizational effects of steroids and changes in permanently infertile ewes is the inability of ovariectomized affected ewes to release a surge of LH in response to oestradiol (Findlay *et al.*, 1973).

The ewes in the present study and those of Adams (1976) and Tang & Adams (1981) had never been exposed to phyto-oestrogens until after puberty. Thus, the permanent effects of phyto-oestrogens on the ewe mimic at least some of the changes caused by hormonal steroids during differentiation, even though the phyto-oestrogens are administered outside the normal period of organogenesis. It is not known whether steroidal oestrogens given to the adult ewe over a long period can also induce permanent differentiation.

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THE OESTROGENIC ACTIVITY OF RED CLOVER ISOFLAVONES AND SOME OF THEIR DEGRADATION PRODUCTS

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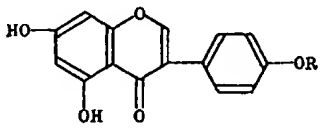
SUMMARY

The oestrogenic activities of the isoflavones biochanin A, genistein, formononetin and daidzein have been measured at three dose levels using the mouse uterine weight assay. The relative activities found were: genistein 1·5, biochanin A 1·0, daidzein 0·4. Formononetin had very little or no oestrogenic activity. The equivalent activity of a sample of Montgomery red clover, expressed in terms of biochanin A, was found to be in good agreement with that expected from concentrations of isoflavones determined from chemical analysis. A study was also made of *p*-hydroxyphenylacetic acid and the benzyl phenyl ketones related to the four isoflavones. These are products of chemical degradation of the isoflavones and were thought to be possible metabolic products of isoflavones in animals. None of these substances were found to have oestrogenic activity.

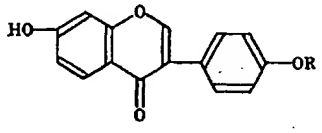
INTRODUCTION

Oestrogenic activities of isoflavones

The isoflavones biochanin A (I), genistein (II) and formononetin (III) are known to be present in red clover (*Trifolium pratense*) as well as in subterranean clover (*Trifolium subterraneum*) and are probably responsible for most of the oestrogenic activity of these pasture species (see reviews of Bradbury & White, 1954; Pope, 1954; Biggers, 1959). Another closely related isoflavone, daidzein (IV), previously found in soya bean as the glucoside, has also recently been detected in red clover and other forages (Guggolz, Livingston & Bickoff, 1961; Wong, 1962).



(I) R=CH_3
(II) R=H



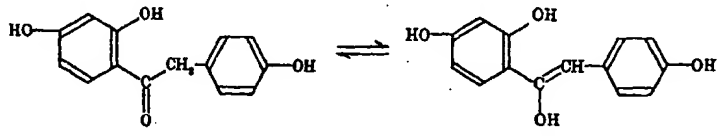
(III) R=CH_3
(IV) R=H

The oestrogenic activities of these isoflavones have been compared with that of diethylstilboestrol by Cheng, Yoder, Story & Burroughs (1954). They found daidzein to be somewhat more active than genistein and biochanin A, and formononetin to have a slight activity. In contrast to these results, Bradbury & White (1954), Pope (1954), and Biggers & Curnow (1954) have found formononetin to be inactive in mice at a variety of dose levels. Bradbury & White (1954) also found daidzein to have no oestrogenic effect in mice at a dose of 5·4 mg. given by injection. Pope (1954) reported that the oestrogenic activity of biochanin A was approx. 0·63 that of genistein.

In order to evaluate the contributions of these constituents to the total activity of red clover, more accurate estimates of their oestrogenic activities are necessary. Also in view of the findings that formononetin and biochanin A are the predominant isoflavones in red clover (Guggolz *et al.* 1961; Wong, 1962) the question of the activity, if any, of formononetin becomes of importance. In this paper the results of a comparative study of the activity of the isoflavones at three dose levels using the mouse uterine weight bioassay are presented, together with the results of a comparison of the oestrogenic activity of a sample of red clover and biochanin A.

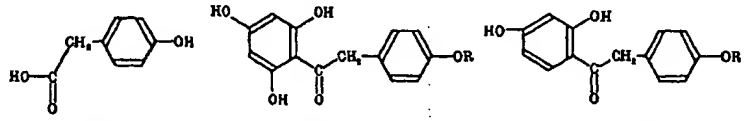
Possible metabolic products of isoflavones in animals

Biggers & Curnow (1954) have reported that genistein behaved as a pro-oestrogen, the oestrogenic activity following its administration probably being the property of a metabolite. The ultimate metabolic fate of isoflavones in animals is unknown. Chemically isoflavones are easily degraded under alkaline conditions to benzyl phenyl ketones (e.g. (V)). These under more drastic conditions can be broken down further to phenols and phenylacetic acids. It seemed possible that isoflavones could be similarly degraded in the animal and that some of these compounds could be oestrogenic. The benzyl phenyl ketones could conceivably enolise to give rise to stilbene-like structures



(V) (VI)

(e.g. (V) ⇌ (VI)) which could be oestrogenic. The increase in oestrogenic activity of plant materials (Beck & Curnow, quoted by Curnow, 1954) and of genistein (Pieterse & Andrew, 1956) after alkaline treatments may have been due to the formation of such compounds. The oestrogenic activities of *p*-hydroxyphenylacetic acid (VII) and the ketones (VIII)–(XI) corresponding to the isoflavones (I)–(IV), respectively, have now been studied.



(VII) (VIII) R=CH_3, (IX) R=H (X) R=CH_3, (XI) R=H

MATERIALS

p-*Hydroxyphenylacetic acid* (VII)

This acid was prepared from *p*-methoxyphenylacetonitrile which was obtained from *p*-methoxybenzaldehyde via the cyanhydrin by the method of Kindler & Peschke (1933). The nitrile (1 g.) and conc. hydriodic acid (S.G. 1·7, 15 ml.) were refluxed for 4 hr., the reaction mixture diluted with water (2 vol.), treated with sodium metabisulphite and the resulting yellow solution extracted with equal volumes of ether (× 3). The ether soluble residue was recrystallized twice in 5 ml. water (charcoal) to give *p*-hydroxyphenylacetic acid (360 mg.), colourless needles, m.p. 153–4° C (Found: C, 63·3; H, 5·2; O, 31·7. Calc. for $C_8H_8O_3$: C, 63·1; H, 5·3; O, 31·5%).

4-Methoxybenzyl 2,4,6-trihydroxyphenyl ketone (VIII)

This substance was obtained from phloroglucinol and *p*-methoxyphenylacetonitrile by the Hoesch reaction, according to Badcock, Cavill, Robertson & Whalley (1950). It crystallized as needles from aqueous alcohol, m.p. 197° C.

4-Methoxybenzyl 2,4-dihydroxyphenyl ketone (X)

This was prepared according to Baker & Eastwood (1929) from resorcinol and *p*-methoxyphenylacetonitrile by the Hoesch reaction. It crystallized as needles from aqueous alcohol, m.p. 159° C (Found: C, 69·1; H, 5·2; OCH_3, 12·1. Calc. for $C_{14}H_{11}O_3$–(OCH_3): C, 69·8; H, 5·5; OCH_3, 12·0%).

4-Hydroxybenzyl 2,4,6-trihydroxyphenyl ketone (IX)

4-Methoxybenzyl 2,4,6-trihydroxyphenyl ketone (VIII) from above (1 g.) was refluxed with aluminium chloride (10 g.) in benzene (100 ml.) for 4 hr. with stirring. Benzene was removed under reduced pressure and ice-cold water (50 ml.) added to the residue followed by an equal volume of 6 N-HCl. The resulting suspension was extracted three times with 100 ml. portions of ether. The ether extract was evaporated and the residue taken up in boiling water and filtered. The filtrate on cooling deposited small needles, indefinite m.p. 246–51° C. Paper chromatography in the solvent system benzene-acetic acid-water (125:72:3) showed that the product contained unchanged starting material. Cellulose column partition chromatography with the above solvent system resulted in the separation of the two components. The product was recrystallized in water, yielding small needles m.p. 260°C (253–7°; Walz, 1931).

Attempts to demethylate (VIII) to (IX) with hydriodic acid (S.G. 1·7) resulted in the formation of phloroglucinol and *p*-hydroxyphenylacetic acid.

4-Hydroxybenzyl 2,4-dihydroxyphenyl ketone (XI)

This was obtained from 4-methoxybenzyl 2,4-dihydroxyphenyl ketone (X) by demethylation with aluminium chloride in benzene as described for (IX) above. The product was purified by chromatography through the same partition column. It crystallized from water as fine needles, m.p. 191–2° C in agreement with that reported by Walz (1931). Treatment of (X) with hydriodic acid resulted in resorcinol and *p*-hydroxyphenylacetic acid.

Biochanin A (I)

This was synthesized from 4-methoxybenzyl 2,4,6-trihydroxyphenyl ketone (VIII) and ethoxalyl chloride by the method of Baker, Chadderton, Harborne & Ollis (1953). Recrystallization from aqueous alcohol yielded needles, m.p. 214° c. Acetate m.p. 191–2° c.

Formononetin (III)

This isoflavone was obtained from 4-methoxybenzyl 2,4-dihydroxyphenyl ketone (X) by the ethyl orthoformate-pyridine method of Sathe & Venkataraman (1949). It crystallized as plates from alcohol, m.p. 256–7° c. Acetate m.p. 171° c.

Daidzein (IV)

Formononetin (800 mg.) was refluxed with hydriodic acid (s.g. 1·7, 15 ml.) for 3½ hr. The mixture after cooling overnight deposited a light-brown precipitate which was filtered off. The dark mother liquor was diluted with 3 vol. water whereby further material was precipitated. The mixture was treated with sodium metabisulphite and filtered. The combined precipitates were recrystallized in alcohol (*ca.* 75 ml.) to yield an off-white powder (520 mg.). Further crystallization in alcohol yielded needles, m.p. 324° c (decomp.). Acetate m.p. 187° c.

Genistein (II)

This was obtained similarly by demethylation of biochanin A, with conc. hydriodic acid (cf. Shriner & Hull, 1945). It crystallized as needles from alcohol, m.p. 296° c (decomp.). Acetate m.p. 202° c.

BIOASSAYS

The mice were from the same colony and were treated in the same way as those used by Munford & Flux (1961). Ovariectomized females 21–23 days of age were allotted at random to treatment groups, six being used at each dose level of each material to be tested.

For all experiments a meal consisting of one part dried buttermilk powder to two parts wholemeal flour was used. Materials to be tested were added to this as follows.

(i) For the bioassay comparisons of oestrogenic activities of pure compounds, these were ground finely and mixed with meal. This was fed at the rate of 2·5 g./mouse/day for 6 days. The quantities of isoflavones added would give total doses of 3, 6 and 12 mg./mouse, respectively, to the mice on the three dose levels. For diethylstilboestrol the total doses were arranged to be 0·036, 0·072 and 0·144 μg./mouse.

(ii) For the comparison of the oestrogenic activity of biochanin A and red clover leaves and petioles, powdered ryegrass, known to be free from oestrogenic activity, was added to the meal containing red clover powder or biochanin A so as to standardize the plant-material content of all the diets at 10%. Red clover was added to the meal to give three total dose levels, planned as 375, 750 and 1500 mg./mouse, respectively, and biochanin A was added to give total dose levels of 5, 10 and 20 mg./mouse. The meal containing these materials was fed in the same way as that in (i).

(iii) For the tests for oestrogenic activity the compounds were added to the meal in the same way as those described in (i) above except that the only dose level used was 12 mg./mouse. These mixtures were also fed at a rate of 2·5 g./mouse/day for 6 days.

The criterion used as an indication of the presence of oestrogenic activity was a statistically significant increase in the mean uterine weight of the treated mice over that of control mice. Transformation of data was not necessary in the tests for oestrogenic activity because the uterine weights of the mice differed little within or between groups and the actual weights could be analysed statistically. In the bioassays, however, where three dose levels of each compound or clover were used and uterine weights were affected by the treatments, the variance increased with the means, making transformation of uterine weights to logarithms necessary. The relationship of $\log_{10}$ dose and $\log_{10}$ uterine weight in each case was linear or close to linear. Relative potencies were calculated according to the methods described by Finney (1950).

RESULTS

Comparison between genistein, biochanin A, daidzein, formononetin and diethylstilboestrol

The results of the experiment in which genistein, biochanin A, formononetin and diethylstilboestrol were compared are shown in Table 1 and those for biochanin A and daidzein in Table 2. The dose rates planned were achieved for diethylstilboestrol and a close approximation was obtained for formononetin, but a falling off of intake as dose of isoflavone increased was seen with genistein and biochanin A.

Table 1. *Uterine and vaginal responses to isoflavones and diethylstilboestrol, and relative potencies of biochanin A, genistein and diethylstilboestrol*

Material	Total dose /mouse (six mice/group)	Mean uterine weight (mg.)	No. of positive vaginal smears
Controls	—	5·9 ± 0·13[a]	0
Formononetin	3·00 mg.	6·1 ± 0·13	0
	5·99 mg.	6·4 ± 0·13	0
	11·96 mg.	6·7 ± 0·13	0
Genistein	2·99 mg.	6·8	0
	5·77 mg.	12·3	5
	11·13 mg.	23·3	6
Biochanin A	2·94 mg.	7·0	0
	5·90 mg.	8·2	4
	11·72 mg.	14·2	5
Diethylstilboestrol	0·036 μg.	7·9	0
	0·072 μg.	9·7	0
	0·144 μg.	16·0	0

Relative activities based on uterine weight response (95% fiducial limits): genistein/biochanin A, 1·49 (0·97–2·29); genistein/diethylstilboestrol, $13{\cdot}5 \times 10^{-4}$ ($10{\cdot}3$–$17{\cdot}8 \times 10^{-4}$); biochanin A/diethylstilboestrol, $9{\cdot}1 \times 10^{-4}$ ($6{\cdot}0$–$13{\cdot}7 \times 10^{-4}$).

[a] S.E.M.'s apply to control and formononetin treated mice only.

Formononetin did not cause significant increases in uterine weight, nor did any of the mice to which it was given show vaginal opening. Genistein and biochanin A caused increase in uterine weight, and, at the higher dose rates, caused most of the mice to show positive vaginal smears. Diethylstilboestrol caused increased uterine weights, but, at the dose levels used, no vaginal responses. The bioassays (Table 1) showed that genistein was the most potent of the isoflavones and biochanin A was

about two thirds as active as genistein. No valid bioassay was possible with formononetin because its dose response line differed too much in slope from the others. Both genistein and biochanin A were much less active than diethylstilboestrol, the ratio of the activities being of the order of 1:100,000.

The activity of daidzein was about two-fifths of that of biochanin A (Table 2) and at the highest dose rate used it caused only one mouse to show a positive vaginal smear although all six showed vaginal opening. In this respect it differed little from the dose of biochanin A which had a similar effect on uterine weight.

Table 2. *Uterine and vaginal responses to daidzein and biochanin A, and potency of daidzein relative to biochanin A*

Material	Total dose/mouse (mg.; six mice/group)	Mean uterine weight (mg.)	No. of positive vaginal smears
Daidzein	3·0	5·8	0
	6·0	7·7	0
	12·0	7·9	1
Biochanin A	3·0	7·7	0
	6·0	13·6	4
	12·0	14·4	5

Activity of daidzein relative to biochanin A, 0·39 (95% fiducial limits, 0·25–0·44).

Table 3. *Uterine and vaginal responses to red clover and biochanin A, and estimated potency of red clover in terms of biochanin A*

Material	Total dose/mouse (mg.; six mice/group)	Mean uterine weight (mg.)	No. of positive vaginal smears
Biochanin A	4·85	6·2	0
	9·20	15·1	5
	18·60	26·6	6
Red clover (leaf and petiole)	360	6·5	1
	670	10·6	1
	1400	17·6	5

Activity of red clover leaves and petioles in terms of biochanin A, 10·1 mg./g. dry clover (95% fiducial limits, 7·6–13·2).

Table 4. *Uterine and vaginal responses to some isoflavone degradation products*

Material*	Mean uterine weight (mg.)	No. of positive vaginal smears
(A) Controls	6·6 ± 0·13	0
4-Hydroxybenzyl 2,4,6-trihydroxyphenyl ketone	6·7 ± 0·13	0
4-Hydroxybenzyl 2,4-dihydroxyphenyl ketone	7·1 ± 0·13	0
p-Hydroxyphenylacetic acid	6·6 ± 0·13	0
(B) Controls	5·1 ± 0·18	0
4-Methoxybenzyl 2,4,6-trihydroxyphenyl ketone	4·7 ± 0·18	0
4-Methoxybenzyl 2,4-dihydroxyphenyl ketone	5·5 ± 0·18	0

* Total dose for each 12 mg./mouse.

10·1 mg. biochanin A/g. dry clover (95% fiducial limits, 7·6–13·2 mg./g.) is in good agreement with concentration of isoflavones as determined from chemical analysis (Wong, 1962). Results for two samples of the same New Zealand Montgomery red clover were 8·4 and 9·4 mg. biochanin A/g. dry clover. The concentration of formononetin was also found to be high, but genistein and daidzein were negligible by comparison. These results from bio- and chemical assays show that the oestrogenic activity of red clover could be mainly accounted for by biochanin A. They also confirm that formononetin contributes little if anything to the oestrogenic activity of the plant material.

The inactivity of the ketones and *p*-hydroxyphenylacetic acid shows that these are not the active metabolites of isoflavones. Pieterse & Andrew (1956) have reported that when genistein was degraded by refluxing for 3 hr. with 2·5% alcoholic potassium hydroxide and the resulting solution fed to mice, about a fourfold increase in oestrogenic activity was found. Curnow (1954) has also quoted evidence that treatment of subterranean clover 'chloroplast' with alkali yields a small amount of a substance with oestrogenic activity at least ten times that of genistein. In view of the inactivity of the *p*-hydroxyphenylacetic acid and the benzyl phenyl ketones which would be expected to be the major products formed after alkaline treatment of isoflavones, the increase in activity found by these workers must be due to some minor products.

The oestrogenic activities discussed here apply to mice. The activities of the isoflavones in ruminants are not known. Nilsson (1961) has shown that transformation of biochanin A to genistein occurs in rumen fluid *in vitro*. If this demethylation can take place with formononetin to give daidzein, and if daidzein is active in ruminants, then the presence of large amounts of formononetin in red clover, although not important with mice, may be significant oestrogenically in sheep.

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Equivalent activity of red clover

The results of the estimation of the oestrogenic activity of a sample of red clover leaves and petioles in terms of biochanin A are shown in Table 3. The activity of the clover was estimated as equivalent to that of 10·1 mg. biochanin A/g. dry clover. Positive vaginal smears were recorded for most mice fed at the highest dose rate.

Oestrogenic activity of degradation products

The results of the tests for oestrogenic activity of *p*-hydroxyphenylacetic acid and the benzyl phenyl ketones corresponding with genistein, daidzein, biochanin A and formononetin are shown in the two parts of Table 4. None caused uterine weights to increase significantly over those of the control mice in the same experiment, nor did they cause vaginal opening.

DISCUSSION

In the present study formononetin was found to have little or no oestrogenic effect when fed to mice in total doses of up to 12 mg./mouse, this result being in accordance with those of Bradbury & White (1954), Pope (1954) and Biggers & Curnow (1954) with similar doses, given by injection. Cheng *et al.* (1954) who used only one dose level, 10 mg./mouse given orally, reported a small increase in the mean uterine weight of treated mice over that of controls. However, the weight of the evidence suggests that formononetin has little or no oestrogenic effect when given by injection or orally to mice in doses of the order of 12 mg. or less.

The estimates of the relative activities of genistein, biochanin A and daidzein of 1·5:1·0:0·4, respectively, differ from those of Cheng *et al.* (1954) who reported daidzein to be more active than genistein or biochanin A. However, those in the present study, being based on more adequate bioassays, are to be preferred. The relative activity of genistein and biochanin A found was in close agreement with that of Pope (1954).

The 95% fiducial limits for the activity of genistein in terms of biochanin A included the value of one, but as this was just within the lower limit it is unlikely that genistein was not the more active compound. The upper 95% fiducial limit for the estimate of the activity of daidzein relative to biochanin A was only 0·44, hence there was little doubt that daidzein was the less active compound in this bioassay.

The estimated activity of genistein in terms of diethylstilboestrol in the present study was about 50% higher than that obtained earlier in the same laboratory (Munford & Flux, 1961) but included the lower estimate within its fiducial limits.

The quantal information of the frequencies of positive vaginal smears was not used for bioassays because of the small numbers of animals involved but served to confirm that the isoflavones genistein and biochanin A were oestrogens or gave rise to oestrogens. The single positive vaginal smear for daidzein was insufficient support for any conclusion on this compound. As was the case with the earlier comparison of the activities of diethylstilboestrol and genistein by Munford & Flux (1961), it appears that the estimated relative activities of the oestrogenic isoflavones and diethylstilboestrol might be different if the occurrence of positive vaginal smears in mice was used instead of increase in uterine weight.

The activity of the red clover sample found here, equivalent to a concentration of

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DETERMINATION OF URINARY LIGNANS AND PHYTOESTROGEN METABOLITES, POTENTIAL ANTIESTROGENS AND ANTICARCINOGENS, IN URINE OF WOMEN ON VARIOUS HABITUAL DIETS

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Summary—Recently two groups of compounds with diphenolic structure, the lignans and the isoflavonic phytoestrogens, were detected and identified in human urine and other biological fluids. These compounds are of great biological interest because they exhibit both *in vitro* and *in vivo* weak estrogenic and sometimes also antiestrogenic activities and many plant lignans have been shown to have anticarcinogenic, antiviral, antifungal and other interesting biological effects. The compounds found in relatively large amounts (10–1000 times more than estrogens) in urine are modified by intestinal bacteria from plant lignans and phytoestrogens, which are present in fiber-rich food such as grain and beans. They bind with low affinity to estrogen receptors and preliminary results suggest that they may induce production of sex hormone binding globulin (SHBG) in the liver and in this way may influence sex hormone metabolism and biological effects. Five compounds, the lignans enterolactone (Enl), enterodiol (End) and the isoflavonic phytoestrogen metabolites daidzein (Da), equol (Eq) and *O*-desmethylangolensin (*O*-Dma) were measured in urine by gas chromatography–mass spectrometry (selected ion monitoring) using deuterated internal standards in 5 groups of women (total number 53). The members of three dietary groups (omnivores, lactovegetarians and macrobiotics) were living in Boston and of two groups in Helsinki (omnivores and lactovegetarians). Until now measurements have been carried out in 94 72-h samples. The highest mean excretion of the most abundant compound, enterolactone, was found in the macrobiotic group and the lowest in the omnivoric groups. Total mean 24-h excretion of enterolactone was 17,680 nmol in the macrobiotics, 4,170 nmol in the Boston lactovegetarians, 3,650 nmol in the Helsinki lactovegetarians, 2,460 nmol in the Helsinki omnivores and 2,050 nmol in the Boston omnivores. The other diphenols followed approximately the same pattern. In an earlier study the lowest excretion of enterolactone (1,040 nmol/24 h) was found in a group of postmenopausal apparently healthy breast cancer patients living in Boston. It is concluded that further studies are necessary to elucidate the possible role of these compounds in cancer and other diseases. However, the evidence obtained until now seems to justify the conclusion that these compounds may be among the dietary factors affording protection against hormone-dependent cancers in vegetarians and semivegetarians.

INTRODUCTION

Since 1979 a number of compounds belonging to two classes of diphenols of great biological interest were detected and identified in human urine and other biological fluids, i.e. the lignans [1–5] and the isoflavonic phytoestrogens [5–8]. Because some evidence suggests that these compounds may be protective with regard to estrogen dependent and perhaps other types of cancer and since many plant lignans have anticarcinogenic, antiviral and fungicidal properties [see 9, 10] we have initiated a series of studies in order to clarify the role of these compounds in human health. The following is a brief summary of the present knowledge with regard to these new compounds and a presentation of preliminary quantitative data on their urinary excretion in five groups of healthy young women consuming their habitual diet. In these studies we also included some vegetarian groups, as it is well known that women living in countries consuming vegetarian or semivegetarian food have a lower incidence of breast cancer [11–18].

LIGNANS IDENTIFIED IN MAN AND ANIMALS

The detection of a cyclic excretion of two unknown phenolic compounds in animal and human urine during the menstrual cycle [1, 2, 19] led to an intense search for, as we believed, new endogenous hormone, but later they were found to be hormone-like compounds of plant origin structurally modified by intestinal bacteria (see below). Two groups of investigators [3, 4] simultaneously presented in *Nature* the structure of these compounds, now called animal or mammalian lignans, to differentiate them from plant lignans. The main compound is now named enterolactone (Enl) [*trans*-2,3-*bis*(3-hydroxybenzyl)-γ-butyrolactone] and its reduction product enterodiol (End) [2,3-*bis*(3-hydroxybenzyl)-butane-1,4-diol]. Recently also the common plant lignan matairesinol (Mat) [*trans*-2,3-*bis*(3-methoxy-4-hydroxybenzyl)-γ-butyrolactone] (Fig. 1), the immediate precursor of Enl was identified in human urine [5]. Both Enl and Mat were identified also in cow milk [20] and in addition End was detected in low amounts in this

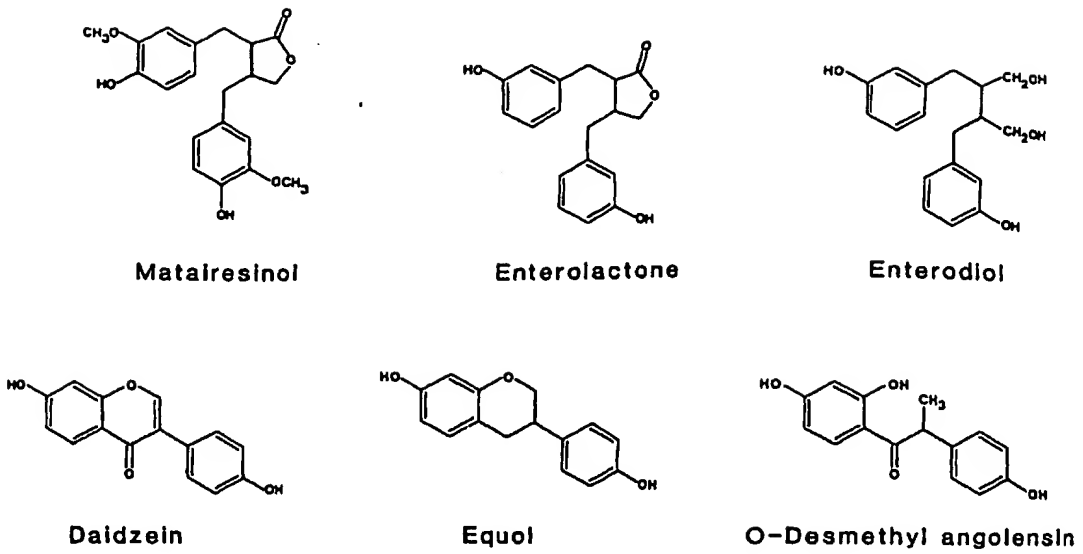

Fig. 1. Structure and trivial names of the mammalian lignans and isoflavonic phytoestrogens identified in human urine.

fluid [21]. Recently we also identified the lignans secoisolariciresinol (the immediate plant precursor of End), lariciresinol and isolariciresinol in human urine (unpublished results).

ISOFLAVONIC PHYTOESTROGEN METABOLITES IDENTIFIED IN MAN AND ANIMALS

Isoflavonic phytoestrogens were the reason for massive outbreaks of infertility in sheep in Australia, when grazing formononetin-containing clover [see 22, 23]. Formononetin (4'-methoxy-7-hydroxyisoflavone) (For) is converted by ruminal bacteria to daidzein (Da) (7,4'-dihydroxyisoflavone), equol (Eq) (7,4'-dihydroxyisoflavane), and *O*-desmethylangolensin (*O*-Dma) [1-(2,4-dihydroxyphenoxphenyl)-2-(4-hydroxyphenyl)propan-1-one] (Fig. 1). Eq is the main product and seems to be responsible for the infertility syndrome. It was first identified in urine of pregnant mares [24] and later also found in the urine of many other animals [23]. Recently Eq [6, 7], Da and *O*-Dma [5, 8] were identified in human urine and we now have evidence for the presence of genistein. Both Eq and a new metabolite methylequol (MeOEq) were identified in cow milk [20]. MeOEq is an intermediate metabolite between For and Eq and represents a second pathway from For to equol in sheep [23]. Furthermore we have recently identified biochanin A, genistein, For, daidzein, MeOEq and *O*-Dma in cow urine (unpublished results).

ORIGIN AND METABOLISM OF LIGNANS AND ISOFLAVONIC PHYTOESTROGENS IN MAN

It has now been demonstrated that human diet, especially grain and other fiber-rich food [10, 25], contains plant lignans, which act as precursors for the mammalian lignans having different structures, the structural modification being carried out by bacteria in the intestinal tract [26–29]. Secoisolariciresinol, which we recently identified in human urine, is one of the precursors and has been found in large amounts in linseed as a glycoside [28]. If linseed is added to the diet of rats or human subjects large amounts of Enl and End are excreted in urine. When Mat, also identified in human urine [5] is added to rat diet, Enl is excreted in urine [28]. In five groups of women the geometric mean excretions of Enl in urine correlated highly significantly with the geometric means of the intake of dietary grain products expressed as kcal/day (Fig. 2) and this is true also if the calculation is done using individual values. Germ-free rats as opposed to conventional rats do not excrete any lignans in urine [30]. Chimpanzees eating their regular diet excrete large amounts of lignans in urine, but on a high-fat diet the urinary lignans almost disappear [31]. Administration of antibiotics results in a dramatic decrease in the excretion of lignans in urine and feces [26] (see also Fig. 3). When End was administered orally to rats, Enl was excreted in urine, but if these experiments were carried out in germ-free rats or if End was administered intraperitoneally to bile-fistula rats, no Enl was detected [28].

The mode of formation of isoflavonic phytoestrogen metabolites in man seems to be similar to that in sheep. Administration of soya protein results in a marked increase in the excretion of Eq, which is a metabolite of Da found as a glycoside in soya flour [32]. Some subjects may not be able to form Eq [32] and germ-free rats do not excrete Eq when given commercial pellet food [30]. *O*-Dma seems to be a minor metabolite in man (see below). Da and Eq are present also in cow milk [20, 21] and it therefore

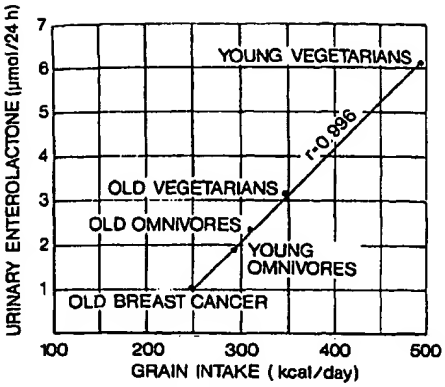

Fig. 2. Correlation between urinary excretion of the lignan enterolactone and dietary intake of grain products (kcal/day) in 5 groups of women consuming their habitual diet. Each group consists of 10 women except the old breast cancer patients ($n = 7$). Each woman collected one 72-h urine sample 4 times with about 3 month intervals during one year and each point represent the geometric means of 40 values corresponding to 120 24-h urine specimens, except for the breast cancer group (84 24-h specimens). The samples in the young women were taken in the midfollicular phase of the menstrual cycle. Enterolactone was assayed by capillary gas chromatography [42]. The results for the postmenopausal women have been published [7].

seems likely that the urinary isoflavonic phytoestrogen metabolites are in man, as in sheep and rat, of dietary origin. Most likely Eq is formed by intestinal bacterial action from Da and perhaps also from For present in food. However, until now we have not been able to detect any For in human urine.

BIOLOGICAL EFFECTS OF LIGNANS AND PHYTOESTROGENS

The lignans and the isoflavonic phytoestrogens all have a diphenolic structure resembling those of the very potent synthetic estrogens stilbestrol and hexestrol. The lignans End and Enl bind only very weakly to rat uterine cytosol [33 and J. H. Clark and H. Adlercreutz, unpublished observation], and have no estrogenic activity *in vivo* in mice [26]. However, recently Jordan [34] demonstrated weak estrogenic activity (stimulation of prolaction secretion and synthesis of progesterone receptor) of Enl *in vitro*. Both Da and Eq have been found to bind to estrogen receptors and to have estrogenic activity [35, 36]. As both Eq and Da as well as Enl show in some experimental conditions weak estrogenic activity they may at certain concentrations act as antiestrogens. However, *in vitro* Enl showed no antiestrogenic properties [34], but *in vivo* Enl inhibited estrogen-stimulated RNA synthesis in rat uterine tissue when administered 22 h before estradiol [37]. In clover disease of the sheep, the ewes are made permanently infertile by estrogenic clover containing For, which is converted to Da and Eq by ruminal microorganisms [22, 35]. Eq has been found to be weakly estrogenic [22], however, the hypothalami of the affected ewes show relative insensitivity to estradiol [36, 38]. Eq competes with estradiol receptor complex for nuclear binding and yet fails to initiate the replenishment of estrogen receptors effectively in the cytoplasm [36], which suggests that this compound can act as an antiestrogen. Furthermore it is of interest that the concentration of Enl in both human and bovine semen is 2.5–25 times higher than in blood plasma [39], that the lignans show a peak excretion in urine in the luteal phase both in the vervet monkey and in women [1, 4, 19] and that their excretion increases in pregnancy [1, 2]. These observations support the view that these compounds may be hormone-like substances and/or that their production is connected to hormone metabolism. It has in fact been suggested that the production of lignans from dietary compounds in rats is dependent on the gonadotrophin level [40].

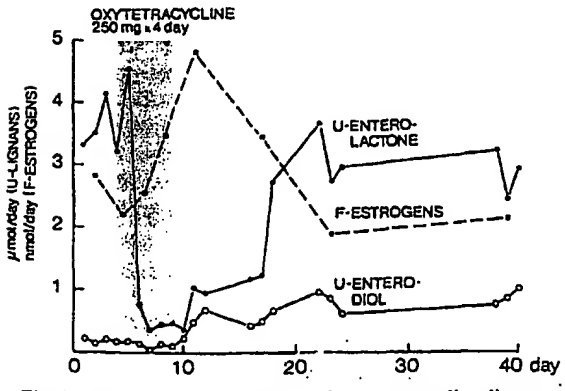

Fig. 3. Mean urinary excretion of the mammalian lignans enterolactone and enterodiol in three young men before, during and after oral administration of 250 mg oxytetracycline 4 times daily. For comparison the excretion of total unconjugated and conjugated estrone + estradiol + estriol in feces is shown.

METHODS USED FOR QUANTITATIVE ASSAYS

Lignans and isoflavonic phytoestrogens have been determined in human and animal urine by capillary gas chromatography [41] and combined gas chromatography–mass spectrometry (GC/MS) [42] utilizing selected ion monitoring (SIM) with or without isotope dilution technique. The sensitivity of capillary GC is not sufficient when the concentrations are low or the amount of sample small, particularly in the case of Eq and Da. *O*-Dma cannot be measured at all by GC. It is of interest to note that Da behaves like a catechol estrogen if anion exchange columns in the borate form are used for purification [43], despite the fact that it does not contain a catechol structure.

In order to measure low amounts of the various lignans and phytoestrogens we have recently further developed the methodology. The new method is outlined in Fig. 4. We synthesized deuterated internal standards of Enl (d_6), End (d_6), Da (d_4), Eq (d_4) and

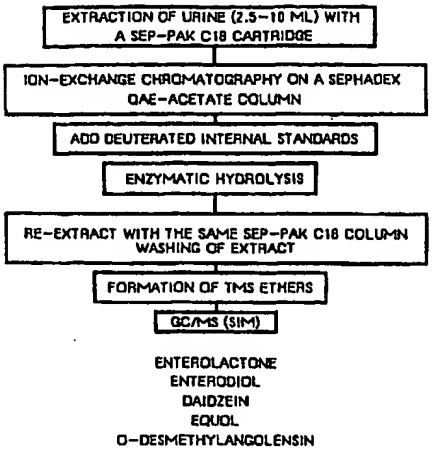

Fig. 4. Flow-diagram of the gas chromatographic-mass spectrometric method used for the determination of lignans and isoflavonic phytoestrogens in urine.

O-Dma (d_3). Because conjugated compounds were not yet available, the deuterated internal standards were added before enzymatic hydrolysis after preliminary purification of the urinary glucuronide conjugates of the lignans and isoflavonic phytoestrogens. It is our intention to try to overcome this problem by modifying the method in order to be able to add the deuterated internal standards in the beginning of the procedure.

DETERMINATIONS IN VARIOUS DIETARY GROUPS

Subjects

Five groups of young women on different habitual diets were investigated. The members of three dietary groups (9 omnivoric, 9 lactovegetarian and 12 macrobiotic subjects, respectively) were living in Boston and of two groups in Helsinki (12 omnivoric and 11 lactovegetarian subjects, respectively). Subjects were excluded if they had a history of any serious disease, diabetes, renal or liver disease, irregular menstrual cycles, use of hormones or regular consumption of alcohol. No samples were taken in 3 months if the subject had taken antibiotics. Careful 3- or 5-day dietary records were made 2–4 times during one year, but these data are not yet available. The women collected 72-h urine samples 2–4 times per year as described previously in another study [44]. Ascorbic acid (0.1%) and sodium azide (0.1%) were used as preservatives. This preliminary report gives the results obtained in 94 72-h urine samples from these 53 women. A more detailed study will be published when the dietary data are available.

RESULTS AND DISCUSSION

The results of the assays expressed as geometric means are shown in Table 1. Of the five diphenols measured the most abundant was Enl followed by Da and End. *O*-Dma occurred in comparatively low amounts except in the macrobiotics who generally excreted very high amounts of all compounds. Excretion of End was usually about 1/10 of that of Enl, only the macrobiotics and to some degree the lactovegetarians in Boston had relatively high excretion of End. This could be due to greater intake of End precursors and/or to a lower conversion of End to Enl in the intestinal tract [27–29]. The ratio of Da to Eq was much lower in the Helsinki women, suggesting greater conversion of Da to Eq by gastrointestinal bacteria compared to the American women. The reason for this may be geographical differences in the quality of the microflora in Finnish and American women which could be due to different composition of the basal diets or even to different contamination of the diet with xenobiotics. As seen in Fig. 3 the effect of antibiotics on urinary excretion of the lignans lasts more than 6 weeks and is characterized by an increased relative amount of End after the initial rapid decrease in the excretion of both Enl and End. Mammalian lignan excretion decreases also in feces (not shown) [26] but for comparison is shown the completely different opposite change in estrogen excretion in feces. It is not known whether small amounts of different xenobiotics contaminating e.g. meat or milk could permanently change the microflora and its biological activities.

In a previous study [7] we reported that the excretion of Enl is significantly lower in the urine

Table 1. Urinary excretion of lignans and isoflavonic phytoestrogens in young women on various habitual diets (geometrical means, nmol/24 h)

	Omnivores	Lactovegetarians	Macrobiotics
Boston women			
Enterolactone	2,050	4,170	17,680
Enterodiol	280	740	6,260
Daidzein	320	1,260	3,460
Equol	69	100	868
O-Desmethylangolensin	33	106	378
Helsinki women			
Enterolactone	2,460	3,650	
Enterodiol	203	368	
Daidzein	219	275	
Equol	102	64	
O-Desmethylangolensin	25	43	

of postmenopausal breast cancer patients (1,040 nmol/24 h) compared to postmenopausal controls eating a normal mixed (2,300 nmol/24 h) or a lactovegetarian (3,180 nmol/24 h) diet (three groups in Fig. 2). The two other groups shown in Fig. 2 are young omnivoric and vegetarian women studied at the same time in Boston using the gas chromatographic method for lignans [41]. The results of these groups have not previously been published, but show slightly lower values for the omnivoric group (1,860 nmol/24 h) than found in the present investigation and somewhat higher in the vegetarian group (6,150 nmol/24 h), the latter high mean value being due to the fact that both lactovegetarians and macrobiotics were included in the vegetarian group.

In the present preliminary study with the GC/MS method in other young women, the lowest mean excretion was found in the omnivoric women living in Boston (2,050 nmol/24 h) who have the highest risk for breast cancer as judged from epidemiological studies. Both the lactovegetarians (4,170 nmol/24 h) and particularly macrobiotics (17,680 nmol/l) in Boston had much higher values. The omnivoric women in Helsinki had 20% higher mean excretion of Enl (2,460 nmol/24 h) than the corresponding group in Boston. The lactovegetarians in Helsinki had 48% higher mean value compared to the omnivores in the same city but had a lower mean excretion (3,650 nmol/24 h) than the lactovegetarians in Boston. In another investigation carried out in North-Karelia (unpublished) where the incidence of breast cancer is lower than in both Helsinki and Boston, the mean excretion of enterolactone in 28 young omnivoric women was much higher (3,020 nmol/24 h) than for any of the other omnivoric groups in Helsinki and Boston. We are at present also measuring these compounds in a group of young Finnish women with breast cancer but the results are not yet available.

Non-human primates are remarkably resistant to the carcinogenic effect of estrogens even in combination with a potent carcinogen. In a recent study we found that in chimpanzees consuming their normal diet the excretion of both lignans and isoflavonic phytoestrogens in urine is very high. The lignan excretion corresponded approximately to that observed in the macrobiotics but the isoflavonic phytoestrogen excretion was about 10 times higher [31]. These findings seem to support our view that these compounds may have protective effect with regard to estrogen-dependent cancer.

One mechanism by which these compounds could influence carcinogenesis is perhaps via a reduction of the sensitivity of estrogen receptors to estradiol as found with regard to hypothalamic estrogen receptors in sheep grazing clover rich in formononetin [36, 38]. In these animals formononetin is converted to large amounts of Eq, which seems to cause this effect. All diphenols are weak antioxidants which may have some positive anticancer effects particularly in the intestine. Furthermore the lignans, including matairesinol, and the three isoflavonic phytoestrogen metabolites identified in human urine have, at high concentrations, antitumor-promotor effects as judged from binding inhibition studies using [^{3}H]12-*O*-tetradecanoylphorbol 13 acetate (TPA) with a mouse skin particulate fraction (T. Horiuchi, H. Fujiki and H. Adlercreutz, unpublished observations). The physiological significance, however, remains uncertain due to the high concentrations necessary (at least 100 μmol/l).

We recently carried out some other analyses including measurements of sex hormone binding globulin (SHBG) and free testosterone in plasma in the Finnish subjects. Furthermore we have expanded the material with some older women and breast cancer patients. We then made a preliminary statistical evaluation of the results obtained hitherto in these groups of women. In the group of 23 women described above we found statistically significant positive correlations between urinary Enl, total lignan, total phytoestrogen and total diphenol excretion, and plasma SHBG. The correlation coefficients were 0.563 ($P < 0.01$), 0.562 ($P < 0.01$), 0.434 ($P < 0.05$) and 0.598 ($P < 0.01$), respectively. In the larger group of women ($n = 67$) we found the same positive correlations between urinary lignans and plasma SHBG but if the Eq values were added to the Enl values the *P*-value changed from <0.01 to <0.001 suggesting that also the phytostrogens may influence SHBG synthesis. In addition we found a negative correlation between urinary Enl and plasma free testosterone ($P < 0.001$). It should be remembered that testosterone stimulates prostatic cancer growth and is the immediate precursor of estradiol.

Previously we have found a positive correlation between dietary intake of total fiber and urinary excretion of Enl [25], and in Fig. 1 a positive correlation is shown between dietary intake of grain (kcal/day) and urinary enterolactone excretion. In the larger material we also found, in preliminary statistical studies, a highly significant positive correlation between intake of dietary total fiber/kg weight and plasma SHBG ($P < 0.001$). These results suggest that fiber-rich food containing lignan precursors may, via production of mammalian lignans in the intestinal tract, stimulate SHBG synthesis in the liver and may in this way reduce the concentration of free hormones in plasma. It is well known that oral estrogens in contradiction to parenterally administered ones markedly stimulate SHBG synthesis [45, 46] and it is therefore not unlikely that these diphenolic weakly estrogenic compounds entering the portal circulation in very high amounts have such a stimulatory effect. This may explain the higher SHBG-values seen in vegetarians consuming fiber-rich food.

It is obvious that further studies are necessary before the possible biological role of these compounds can be defined. However, it seems justified to conclude that these compounds may be one of the dietary factors protecting those consuming a vegetar-

ian or semivegetarian diet against the development of hormone-dependent cancer. Much evidence has in fact been collected suggesting that other dietary phenolic compounds inhibit neoplasia [47]. We have also suggested that lignans may be protective with regard to other types of cancer such as colon cancer [10, 26], but this has not been discussed in this communication.

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E15

V/1 Occurrence of Anabolic Agents in Plants and their Importance

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Summary

More than 40 plant species have been shown to contain substances that are active in biological assays for estrogenic activity. Such substances may be constitutive metabolic products of a plant, or be formed adaptively in response to environmental factors, such as fungal attack (e.g. coumestrol synthesis in alfalfa infected with *Pseudopeziza medicagensis*); in other instances estrogens may arise from microbial attack on plant material during storage (e.g. zearalenone formation from corn by *Fusarium* spp.). Phyto-estrogens may reach man through direct consumption of fresh fruit, vegetables and processed plant products (e.g. administration of olive or cornoil can induce vaginal keratinization in post-menopausal women); or – more relevant to this Symposium – by consumption of carcasses and products from animals fed estrogen-containing forage.

Important pasture and forage plants shown to contain phyto-oestrogens include *Trifolium subterraneum L.*, notably the cultivars Dwalganup, Mt. Barker, Yarloop and Marrar, *T. pratense* (red clover), *T. fragiferum L.* (strawberry clover), *T. alexandrinum* (berseem clover), *Medicago sativa* (alfalfa or lucerne) and *Soya hispida* (soya beans). A beneficial anabolic action of the estrogens contained in these plants has been implied, but not unequivically established. More attention has been paid to their noxious effects on livestock. On affected *T. subterraneum* pasture, castrated male sheep showed lactation, squamous metaplasia of the bulbo-urethral glands and urethral stenosis; infertility, variously attributed to suppression of gonadotrophin release and ovulation; faulty ovum transport; premature regression of corpora lutea; irreversible cystic hyperplasia of endometrial glands on prolonged exposure; dystocia and prolapse of the uterus. Sporadic incidence of phyto-estrogen induced infertility in cattle has been reported, attended by ovarian cyst formation. Estrogenic activity in forage plants has been reported from Australia, New Zealand, India, Sweden, Great Britain, Germany, Denmark, Holland, Finland, Egypt and Israel.

The clover constituents chiefly incriminated for these effects are glycosides of the isoflavone derivatives genistein and its 4'methyl ether biochanin-A, daidzein and its 4'-methyl ether formononetin, and pratensein; coumestrol and its 3'- and 4'-methyl ethers account for the estrogenic activity of alfalfa. The isoflavone content of subterranean clover may reach 3 percent of its dry weight, and the coumestrol content of lucerne may exceed 100 μg/g. Coumestrol and genistein compete with 17β-estradiol for binding sites on the uterine cytoplasmic receptor and induce macromolecular synthesis in the uterus, but fail to induce ovum implantation in ovariectomized, gestagen-maintained rats. Uterotrophic activity of coumestrol and genistein given parenterally to sheep is approximately 10^{-3} and 10^{-5} times that of stilboestrol, respectively. Biological activity of ingested phytoestrogens is modified by ruminal micro-organisms and hepatic metabolism. The pro-estrogens 4'methylcoumestrol, biochanin-A and formononetin undergo *O*-demethylation in the rumen and liver to give rise to coumestrol, genistein and daidzein, respectively. Daidzein is further metabolized in the rumen to equol (about 70 percent) and *O*-desmethylangolensin (5–20 percent), both of which possess weak but significant estrogenic activity; genistein and biochanin-A are transformed chiefly to hormonally inert *p*-ethylphenol. The greater part of the circulating phyto-estrogens occur as glucuronide conjugates. Limited data indicate that estrogenic isoflavones and coumestans accumulate in fat depots in sheep grazing affected pasture, but the amounts reported (about 1 p.p.m.) seem too low to present a significant

health hazard to the human consumer. However, no information on the pharmacology of these substances in primates is available.

In addition to biological screening techniques, specific methods have been developed for chromatographic separation of phyto-estrogens and their determination by spectrophotometry and fluorometry, receptor radioassay or radioimmunoassay. Control measures under investigation include pasture management, selection of isoflavone-deficient mutants and active vaccination with synthetic isoflavone derivatives coupled covalently to polypeptide carriers.

1. Introduction: ecological considerations

Close to 50 plant species have by now been shown to contain substances that are active in biological assays for estrogenic activity conducted in laboratory rodents or in ruminants (for reviews see refs. 10, 15, 46, 79). Such substances may be constitutive metabolic products of the plant, or be formed adaptively in response to environmental factors, such as fungal attack. Thus coumestrol synthesis is induced or greatly augmented in alfalfa (*Medicago sativa*) on infection with the leaf-spotting organism *Pseudopeziza medicagensis*[12,70]. In other instances estrogens may arise from microbial attack during faulty storage, e.g. zearalenone is formed in corn contaminated with *Fusarium spp.*[19]. The ecological factors that determine the balance between estrogenic and non-estrogenic species in natural pastures and the remarkable seasonal and geographical variation in the estrogen content of a given clover strain have not been fully identified[10]. They include among others, the level of phosphate fertilizer applied[3,62].

Oestrogenic activity may occur in bulbs (e.g. *Allium sativum L.*[30] or tubers (e.g. *Butea superba Roxb.*[64], or be localized in the leafy parts of the plant, in its fruit or in its seed[15]. Such phyto-estrogens may reach man through direct consumption of fresh fruit, such as apples[63] and cherries[27,63], vegetables (e.g. potatoes[15]) or condiments such as garlic[30]; from hops used for beer production[90] and other processed plant products; or by consumption of carcasses and products from animals fed estrogen-containing forage. There is no published evidence that herbal estrogens reaching the human from any of these sources is of pathogenic significance. It is of interest, however, that administration of corn oil or olive oil, at the rate of 100 g per day over 10 days, was shown to cause extensive keratinization of the vaginal epithelium in post-menopausal women[84], indicating that herbal estrogens contained in these products are indeed biologically active in man and effectively absorbed from the gut.

2. Distribution and chemical nature

Important pasture and forage plants shown to contain phyto-oestrogens include *Trifolium subterraneum L.*, notably the cultivars of Dwalganup, Mt. Barker, Yarloop, Clare, Geraldton, Dinninup, Woogenellup and Marrar [8,9,13,14,25,41,43], *T. pratense* (red clover[40,87,88]), *T. fragiferum L.* (strawberry clover[61]), *T. alexandrium* (berseem clover[35,68,69]), *T. repens* (Ladino clover[55]), M. sativa (alfalfa or lucerne[11,70] and *Soya hispida* (soya beans[20,69,86]). Only in very few cases were plant estrogens found to be identical with one of the estrogenic hormones of mammals. Examples are the isolation of estrone from palm kernels by Butenandt[18], and the reported occurrence of estriol in willow catkins[78]. With these esoteric exceptions, however, the phytoestrogens thus far identified proved to be phenols not chemically related to the hormonal steroids, but they share with 17β-estradiol certain structural features (Fig. 1) that may account for their biological activity.

The clover constituents chiefly associated with estrogenic activity are glycosides[8] of the isoflavone derivatives genistein[14] and its 4'-methyl ether biochanin-A[58], daidzein and its 4'-methyl ether formononetin (Fig. 2; ref.[15,16,41,43,44,72]) and pratensein[88]; coumestrol

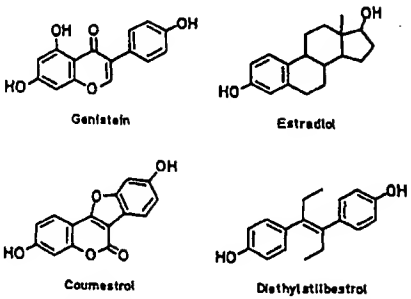

Fig. 1. Structural similarity between non-steroidal estrogens and estradiol

and its 3'- and 4'-methyl ethers account for the estrogenic activity of alfalfa and medics[10,11,16,70,75]. The isoflavon content of subterranean clover may reach 3% of its dry weight[41] and the coumestrol content of lucerne may exceed 100 μg/g[10,69]. The uterotrophic activity of coumestrol and genistein given parenterally to sheep is approximately 10^{-3} and 10^{-5} times that of stilbestrol respectively[16]. Both compounds were also active by the intraruminal route, but potency was 1/20 (genistein) to 1/100 (coumestrol) of that observed on intramuscular administration of the same compound[16,41].

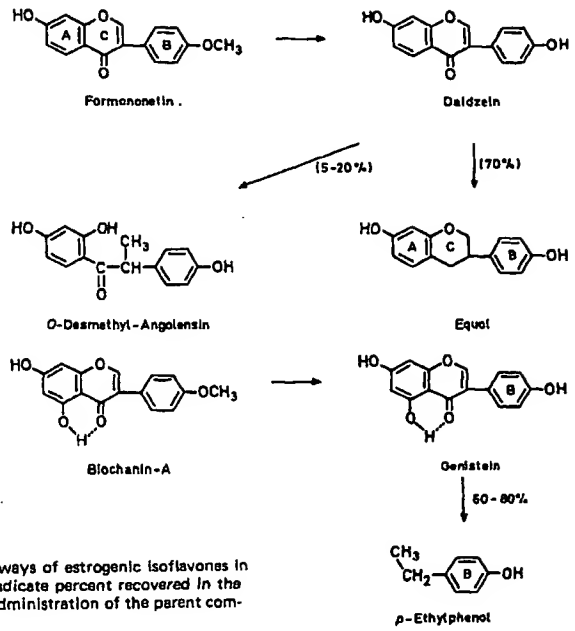

Fig. 2. Major metabolic pathways of estrogenic isoflavones in sheep. Numbers in brackets indicate percent recovered in the urine following intraruminal administration of the parent compound (ref.[72])

A recent analysis of five samples of soybean oil cake (Shemesh, Ayalon and Lindner, unpublished observations), an important ingredient of dairy-cattle diets, showed the presence (p.p.m. in dry matter ± S.E.M.) of daidzein (30.0 ± 4.7), formononetin (4.3 ± 0.2), genistein (18.6 ± 2.7), coumestrol (16.5 ± 2.9) and 4'-methylcoumestrol (0.3 ± 0.02). Berseem clover (*T. alexandrium*), which plays an important part as a cattle feed in the Middle East, was shown by bioassay to contain estrogenic activity[35,63]. Chemical analysis proved that it contains the isoflavones genistein, biochanin-A and formononetin, as well as the coumestan derivative coumestrol (Shemesh, Ayalon and Lindner, unpublished observation); the plasma of heifers fed this forage contained, in addition, daidzein, probably an *O*-demethylation product of formononetin (v.i.).

3. Mode of action

The primary interaction of estradiol with its target cells appears to involve the binding of the hormone to a cytoplasmic protein receptor, characterized by a sedimentation constant of 8S in low-salt sucrose gradients. Both coumestrol and genistein compete with 17β-estradiol for binding sites on this receptor in uterine cytosol preparations from rabbit[71] or sheep[76], but the affinity of the phyto-estrogens is considerably below that of estradiol. The affinity of their methylated derivatives (e.g. formononetin and 4'-methylcoumestrol) for the receptor is still lower, though these compounds are estrogenically active *in vivo*. Isoflavan-7,4'-diol (equol) almost equals genistein in affinity for the receptor. The significance of this compound will be discussed later in relation to the metabolism of formononetin in ruminants.

Coumestrol and genistein also simulate estradiol in stimulating macromolecular synthesis in the uterus[51]. In particular, both phyto-estrogens stimulate the *de novo* synthesis of a specific "estrogen-induced protein", demonstrable within 1 h in the cytoplasm of the rat uterus by double-labelling techniques and gel-electrophoresis (A.M. Kaye, D. Sheratzky-Sömjen and H.R. Lindner, unpublished observations). This protein[52] is considered crucial for the action of estradiol[6]. While both coumestrol and genistein exert uterotrophic activity in the rat, neither compound is able to induce ovum implantation in ovariectomized gestagen-maintained rats, suggesting that the latter response involves a different receptor mechanism[57].

4. Metabolism in grazing animals: role as pro-estrogens

The biological activity of the ingested isoflavones and coumestans is modified by ruminal microorganisms and hepatic metabolism. 4'-Methylcoumestrol, biochanin-A and formononetin undergo *O*-demethylation in the rumen and liver to give rise to coumestrol, genistein and daidzein, respectively (Fig. 2, p. 159; ref.[41,50]). Daidzein is further metabolized in the rumen to equol (about 70%) and *O*-desmethylangolensin (5–20 %), both of which – notably equol – possess weak but significant estrogenic activity; genistein and biochanin-A are transformed chiefly to hormonally inert *p*-ethylphenol (Fig. 2; ref.[16,72,73,74]). Formononetin is also reduced without prior demethylation to 4'-*O*-methyl-equol, which appears in the urine following administration of formononetin to sheep (Cox, Braden and Lightfoot, personal communication). The different metabolic patterns of the 5-hydroxy and 5-deoxy isoflavones probably account for the observation that the estrogenic effects of clover pastures on grazing sheep are more closely correlated with their formononetin than with their genistein content[43,44], in spite of the greater estrogenicity of genistein in parenteral assays in laboratory rodents and sheep[16]. Formononetin and the various methylated derivatives of genistein and coumestrol are thus properly classified as pro-estrogens.

The greater part of the circulating phyto-estrogens in sheep occur as water soluble glucuronide or sulphate conjugates[73].

5. Anabolic action

A beneficial anabolic action of plant estrogens on grazing animals has been implied, but not unequivocally estabilshed. The evidence for this view is, in the main, indirect: castrate lambs grew faster when fed estrogen-containing alfalfa, or when given alfalfa extracts containing coumestrol, than those raised on a non-estrogenic diet[56]. Intact female lambs did not show this response. Again, the growth response to stilboestrol is diminished or abolished in animals consuming estrogenic pasture. This was interpreted to indicate that such animals already receive maximal estrogen-mediated anabolic stimulation from their plant diet[33]. These reports are suggestive but not conclusive, if only because dietary factors other than the plant estrogens may have confounded the results. Equally inconclusive are reports attributing the so-called "spring flush" in milk yield to the estrogen content of pasture[4,5,32,59], or the finding that the plant estrogen coumestrol enhanced the tenderness and juiciness of lambs as judged by a chewing panel[33].

6. Noxious effects on livestock

Much more attention has been paid to the noxious effects of phyto-estrogens on farm animals, and more critical work has been done in this area. This problem was first recognized in 1946 when Bennets and Underwood[9] described massive outbreaks of infertility in sheep grazing subterranean clover in Australia. Subsequently, genistein was isolated from this clover[14]. "Clover disease" is still regarded as one of the major problems of livestock production in Australia[42,45], with about 9 million sheep at risk, and in its milder form it has been recognized as a breeding problem in many other countries. Estrogenic activity in forage plants has been reported in New Zealand[48], the Phillipines[2], Japan[55], The United States of America[10], Canada[37], Chile[31], Central Africa[82], India[36], Egypt[68], Israel[1,70], The Union of Soviet Socialist Republics[77], Finland[34,81], Sweden[49], Denmark[47], Great Britain[59,60], Belgium[26], Holland[85], Germany[65,67], Italy[23] and Czechoslovakia[21,22].

The biological effects of clover estrogens responsible for fertility impairment appear to be multiple. Ewes exposed to affected subterranean clover pastures show mild to severe degrees of infertility, attributed to faulty sperm and ovum transport and interference with ovum implantation[46], premature regression of corpora lutea[53] or cystic hyperplasia of the endometrial glands, leading to irreversible sterility on prolonged exposure[9,46]. Other disturbances include maternal dystocia and prolapse of the uterus, often followed by gangrene in the field[9]. Phyto-estrogens may suppress gonadotrophin secretion[39], possibly by interfering with the positive feedback effect of endogenous estradiol on the hypothalamus (J. Goding, personal communication). Castrated male sheep grazing estrogenic *T. subterraneum* pastures showed lactation, squamous metaplasia of the male accessory glands, at times with gross enlargement of the bulbo-urethral glands and urinary retention[9,46]. The possibility that clover estrogens may cause seminal degeneration in rams has been considered on indirect evidence[46]. Sporadic incidence of phyto-estrogen-induced infertility in cattle has been reported, usually attended by ovarian cyst formation and occasionally by nymphomania[1,23,54,60,66,80,83,89].

7. Residues in animal carcasses

Limited data[41] indicate that estrogenic isoflavones (genistein, biochanin-A, formononetin and daidzein) accumulate in fat depots in sheep grazing estrogenic pasture or given synthetic isoflavones by intra-ruminal infusion. The amounts found in the adipose tissue (about 1 p.p.m.) exceeded the concurrent plasma concentration. Nevertheless, the concentrations reported appear too low to present a serious health hazard to the human consumer, considering the low estrogenic potencies of these substances (cf. ref. 79). However, no infor-

mation on the pharmacology of the herbal estrogens in primates is available. The possibility that such estrogens may pass into milk or milk products should also be kept in mind.

8. Methods of detection

Biological assays, using the uterine weight or vaginal cornification response in ovariectomized laboratory rodents or ewes, or the teat growth response in castrated male sheep, have been found useful for screening purposes[16,17,38,46]. The species used (ruminants vs. monogastric animals) and the route of administration (oral vs. parenteral) may markedly affect the estimate of relative potency obtained, and due regard must also be paid to low solubility of the isoflavones and coumestans in neutral aqueous media.

Paper- thin-layer and gas-chromatographic micromethods are available for the separation of all the known phyto-estrogens from plant material, body fluids and animal tissue[10,28,41,57] and for their quantitative determination by spectrophotometry and fluorometry[10,41], flame-ionization detection[41] or receptor radioassay[71]. Another feasible approach is radioimmunoassay[7]. Receptor radioassay will not distinguish between the endogenous mammalian estrogens and nonsteroidal phyto-estrogens or stilboestrol, and will not detect the methylated herbal pro-estrogens. Immune sera, generated with isoflavone or coumestan haptens covalently attached to a protein carrier, will discriminate between steroidal and isoflavone-derived estrogens, and can be used to measure the active phenols as well as their *O*-methyl ethers[7].

9. Control measures

Control measures under investigation include pasture management designed to preserve a favourable balance between estrogenic legumes and grass[3,10,46,62]; measures to limit fungal infection of forage plants[12]; selection of isoflavone deficient clover mutants[29], which should have desirable agronomic properties, such as the ability to compete with the wild type in the field; and active vaccination with synthetic isoflavone derivatives coupled covalently to polypeptide carriers[7]. High titres of specific antibodies to phyto-estrogens are maintained in sheep for more than a year after primary immunization, are transferred to lambs through the colostrum and do not interfere with the action of endogenous hormones or breeding performance[24]. The protective value and economic feasibility of such vaccination remains to be established, and a more acceptable adjuvant than CFA, which is currently used, may have to be found. If effective, this method would provide the first example of immunization against a naturally occuring disease with a fully synthetic antigen, and as such could be of more general interest.

10. Concluding remarks

Estrogenic substances are widely distributed among plants serving as animal fodder or grown for direct human consumption. By and large they are nonsteroidal compounds capable of interacting directly with the cytoplasmic estrogen receptor by virtue of a structural resemblance to estradiol, – or apt to be metabolized to such estrogenomimetic compounds. It is likely that these compounds possess anabolic activity, but this aspect needs more critical documentation, preferrably including trials with purified substances. In excessive amounts herbal estrogens clearly have adverse effects on reproductive performance in sheep, and more sporadically in other ruminants. Some control measures under active investigation were briefly discussed.

Residues of these estrogens may accumulate in the carcass, and methods are available for their detection, but there is no information to suggest that they present a serious health hazard to man. The ubiquitous background of herbal and endogenous estrogens in animal products may have to be considered when designing regulatory measures and forensic assay procedures to control the use of synthetic anabolic agents. Furthermore, the possible presence of phyto-estrogens in the control diet of experimental animals should be kept in mind when examining the effectiveness of other anabolic agents.

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V/2. Endogenous Anabolic Agents in Farm Animals

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Summary

This presentation is limited to the three groups of steroid sex hormones which alone or in combination have been shown to be anabolic when used in farm animals. It seems essential for realistic evaluation of public health aspects of use of these hormones that the discussions include *naturally occurring* levels of the hormones.

The following topics will be dealt with for each group of hormones: 1. Types and sources; 2. Production rates; 3. Plasma levels; 4. Tissue concentrations; 5. Metabolism and excretion.

Gestagens

Progesterone and 20-dihydroprogesterones are mainly produced in ovaries and placenta. Production rates are estimated to 10 and 14 mg/24 hrs in pregnant goats and sheep, respectively. Plasma levels during the luteal phase are of the order of 2–10 ng/ml, during pregnancy somewhat higher. Muscular tissue from calves contain 0.25 ng/g. In dairy cows progesterone is excreted with the milk which contains up to 30 ng/ml; butterfat up to 300 ng/g. In ruminants progesterone is metabolized mainly to androgens excreted with faeces. In pigs large parts are metabolized to pregnanediols excreted with urine.

Androgens

Testosterone is mainly secreted by testes. Boar testes also produce large amounts of dehydroepiandrosterone and its sulphate. Production rates have been estimated to be 10 mg and 40–50 mg/24 hrs. in boars and bulls respectively. Plasma levels in bulls and rams are generally 2–10 ng/ml, in boars 2–25 ng/ml. Adipose tissue levels up to 22 ng/g are reported for bulls. In ruminants epitestosterone seems to be a major metabolite excreted mainly with faeces. In boars, urinary 11-deoxy-17-ketosteroids are major metabolites of testicular dehydroepiandrosterone. Castration shows elimination to be rapid.

Estrogens

17β-Estradiol and estrone are produced in ovaries and placenta and, in large amounts, in boar and stallion testes. Production rates in late pregnancy are estimated to 10 mg oestrone/24 hrs. in goats, 2 mg estrone and up to 28 mg 17β-estradiol/24 hrs. in sheep. In cows much higher values are found. Boars and stallions produce huge amounts daily. Plasma levels in non-pregnant animals are at the pg/ml level. In late pregnancy levels of 2–4 thousand pg/ml are encountered in sows and cows, in sheep and goats lower levels. Calf muscular tissue contains up to 410 and 610 pg/g of estrone and 17β-estradiol respectively. In muscle from pregnant heifers corresponding values were 120 and 860 pg/g in the 4th month and 2100 and 370 pg/g in the 9th month of pregnancy. Ruminants in large measure metabolize 17β-estradiol and estrone to 17α-estradiol which posesses low estrogenic activity. In pigs estrone dominates in blood and urine. Major routes of elimination are with faeces in ruminants, with urine in pigs and horses. Elimination rates are high.

Results obtained during the last few years clearly show that all three groups of steroid sex hormones occur in considerable concentrations in plasma and tissues. Realization of claims of zero tolerance levels for these compounds is therefore impossible. The problems may be different when compounds other than those occurring naturally are considered.

Urinary excretion of lignans and isoflavonoid phytoestrogens in Japanese men and women consuming a traditional Japanese diet[1-4]

Herman Adlercreutz, Hideo Honjo, Akane Higashi, Theodore Fotsis, Esa Hämäläinen, Takeshi Hasegawa, and Hiroji Okada

ABSTRACT Epidemiologic studies revealed low mortality in hormone-dependent cancer in Japanese women and men consuming a traditional diet. We previously found that certain diphenolic food components, lignans and isoflavonoids, which are converted to biologically active hormone-like substances by intestinal microflora, may be cancer-protective agents. Therefore, we studied urinary excretion of these compounds (enterolactone, enterodiol, daidzein, equol, and *O*-desmethylangolensin) in 10 women and 9 men in a rural village south of Kyoto, Japan. The subjects consumed a typical low-fat diet with much rice and soy products, fish, and vegetables. An isotope-dilution gas chromatographic-mass spectrometric method was used for the assays. The urinary excretion of lignans was low but that of the isoflavonoids was very high. The excretion of isoflavonoids correlated with soybean-product intake. The low mortality in breast and prostate cancer of Japanese women and men, respectively, may be due to the high intake of soybean products. *Am J Clin Nutr* 1991;54:1093–1100.

KEY WORDS Japanese, diet, urine, lignans, isoflavonoids, enterolactone, enterodiol, daidzein, equol, genistein, *O*-desmethylangolensin, soybean, gas chromatography, mass spectrometry, sex-hormone-binding globulin

Introduction

Mammalian lignans and isoflavonoid phytoestrogens, occurring in all studied animal and human biological fluids and in feces, are diphenolic compounds with molecular weights similar to those of steroid estrogens (1–3). Precursors in plants seem to occur as glycosides (4, 5), and the mammalian compounds are produced from plant lignans and isoflavonoids by intestinal microflora (6–8). Most of the original plant aglycones, such as formononetin, matairesinol, and secoisolariciresinol, occur only in very low concentrations in urine (9, 10). All compounds investigated so far are weakly estrogenic but have shown many other biological activities, producing antiestrogenic (1–3); antiviral (11, 12); and antiproliferative, cytotoxic, and growth-inhibiting effects (3, 13–15). Studies indicate that they most likely stimulate the production of sex-hormone–binding globulin (SHBG) in the liver (2, 14–18) and may in this way significantly influence biological activity of the sex hormones. The higher SHBG values seen in vegetarians (2, 17–19) are probably due to the effect of these diphenolic compounds on liver synthesis of the protein (14). Studies in both young and old women with breast cancer and in various dietary groups indicate that urinary excretion of these compounds is highest in vegetarians and lower in omnivores and breast-cancer patients (2, 18, 20). It was shown that their urinary excretion correlates with the intake of fiber-rich food (2, 17, 18).

Japanese women and women of Japanese origin in Hawaii consuming a diet similar to the original traditional Japanese diet have low breast-cancer incidence and mortality (21–24). Similarly, Japanese men have low mortality with prostate cancer, although autopsy studies have found that the incidence of prostate cancer in Japanese and Western men are similar (25–27). These cancers are sex-hormone dependent and could potentially be influenced both by alterations of sex-hormone metabolism caused by lignans and isoflavonoids or by a direct effect of these compounds on their growth. Because of the associations between diet and these diseases, we decided to study the urinary excretion of lignans and isoflavonoid phytoestrogens in groups of Japanese men and women consuming a traditional diet. A preliminary report was published as an abstract (28).

Subjects and methods

Participants

The subjects participating in this investigation were apparently healthy and were recruited in a small rural village south of Kyoto,

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[2] Preliminary report published as an abstract.

[3] Supported by Sigrid Jusélius and Finnish Cancer Foundations and the Medical Research Council of the Academy of Finland.

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Japan. Two of the women were found to have hypertension (blood pressure 146/96 and 180/100, respectively). Most of the participants were farmers cultivating tea and rice. Originally 10 men and 10 women volunteered for the study, but 1 man was dropped because his urine volume was not known. Their main work was in agriculture and they consumed mainly their own products. The ages of the men and women were 50.4 ± 18.0 and 46.8 ± 11.5 y, respectively. Height, weight, and body mass index [BMI, in weight (kg)/height (m)^2] were, respectively, 160.8 ± 7.8 cm, 58.6 ± 5.8 kg, and 22.7 ± 2.3 for men and 153.1 ± 6.5 cm, 52.9 ± 7.2 kg, and 22.6 ± 3.5 for women. All subjects were within 15% of normal weight.

Collection of samples

Urine was collected for 48 h in plastic bottles containing 2 g ascorbic acid. The bottle was kept in a cool place during collection. The urine was mixed and measured and a sample was frozen as soon as possible and transported to Finland in dry ice for analysis.

Dietary data

The study was carried out in October 1985. Before the survey a nutritionist explained how to weigh the food components and how to write down the results on a form. Most of the food was weighed. Some food, such as bread and milk, was recorded as a piece of bread or cup of milk and the nutritionist estimated the weight of these food items afterwards. Food intake was recorded for 3 d and the nutritionist followed all subjects every day during the survey period. Calculation of the food data was made by an experienced nutritionist using the *Standard Tables of Food Composition in Japan* (29); for fiber calculations the *Food Composition Tables of Dietary Fibers, Minerals, Cholesterol, Fatty Acids* was used (30). The amount of soy sauce in the diet was calculated from the total sodium chloride content of the urine. According to earlier studies Japanese obtain 25.8% of their sodium chloride from soy sauce (31). Soy sauce contains 15% NaCl. The consumption of soy sauce is estimated by using the following formula:

$$\text{Soy sauce} = (\text{amount of NaCl in urine}) \times 0.258/0.15$$

This is the traditional way to estimate soy sauce consumption in Japanese subjects because they do not add any other salt to their food. It is an estimate and not an exact figure and the values were not included in the correlation analyses.

Analytical method

The trivial and systematic names of the compounds measured and discussed are as follows [structures were shown previously (3)]: enterolactone (Enl), *trans*-2,3-bis[(3-hydroxyphenyl)methyl]-γ-butyrolactone; enterodiol (End), 2,3-*bis*[(3-hydroxyphenyl)-methyl]-butane-1,4-diol; daidzein (Da), 4',7-dihydroxyisoflavone; equol (Eq), 4',7-dihydroxyisoflavan; *O*-desmethylangolensin (*O*-Dma), 1-(2,4-dihydroxyphenyl)-2-(4-hydroxyphenyl)-propan-1-one.

The method used was a modification of a method for determining the estrogen profile in urine by ion-exchange chromatography and capillary gas chromatography–mass spectrometry in the selected ion-monitoring mode (GC-MS-SIM, or GC/MS) (32–34). Originally, estrogens also were determined but because of very low concentrations of some fractions, the amount of urine saved for the purpose was too small and the analyses could not be repeated. Therefore, only the lignan and isoflavonoid values are presented. Only modifications of the method are described.

Protection of the carbonyl functions by ethoximation (necessary only for the estrogens), and extraction with a Sep-Pak C_{18} cartridge (Waters Associates, Milford, MA) were carried out as described (33, 34). The removal of inhibitors of the enzyme hydrolysis by ion-exchange chromatography on a DEAE-Sephadex (Pharmacia Fine Chemicals, Uppsala, Sweden) column in the acetate form was done in a smaller column (0.5 × 3 cm instead of 0.5 × 5 cm). For hydrolysis and purification of the hydrolysate, before evaporation of the last fraction obtained from the above DEAE-Sephadex column, the following deuterated internal standards were added to the eluate: d_6-Enl and -End, d_4-Da and -Eq, and d_5-*O*-Dma (35, 36). This was followed by hydrolysis and Sep-Pak extraction; application of the methanolic extract directly on the QAE-Sephadex A-25 in the acetate form (0.5 × 5 cm); and elution of the estrogens, lignans, and Eq with 4 mL methanol as described. The modification in this step is that *O*-Dma and Da are eluted after this with 4 mL 0.2 mol acetic acid/L in methanol. This fraction is then, after evaporation of the solvent, ready for derivatization (trimethylsilyl ethers) and GC/MS. Selective fractionation of estrogens with vicinal *cis*-hydroxyls was carried out in a borate column with new dimension (0.5 × 3 cm instead of 0.5 × 2.5 cm). Elution of the diphenols was carried out as described and this fraction contains the isoflavan Eq and the two lignans Ent and End.

The two fractions containing lignans and isoflavonoid phytoestrogens and their deuterated internal standards are converted to their trimethylsilyl ether (TMS) derivatives (32) and quantified by GC/MS by using the following ion pairs (mass/charge): Eq, 386/390; Da, 398/402 (and 383/387); End, 410/416; Enl, 442/448; and *O*-Dma, 459/464 (36). The measurements were carried out with a Hewlett-Packard 5995 B GC/MS (Avondale, PA) instrument equipped with a Pascal work station and with an automatic injector.

Urinary excretion of < 0.0025 μmol/d cannot be measured, and between 0.0025 and 0.005 μmol/d the method must be regarded as semiquantitative. The mean values and interassay imprecision for the control pooled-urine sample, measured 59 times in single assays during 1 y, were as follows: Enl, 3.65 μmol/d (CV 7.4%); End, 0.364 μmol/d (CV 11.6%); and Eq, 0.042 μmol/d (CV 9.4%). For Da at a concentration of 0.028 μmol/d, the interassay imprecision is 11.0% (n = 14) and for *O*-Dma at the high concentrations in this study, the interassay imprecision is 8–10% (CV).

The samples were analyzed in two batches and the values for the control sample were almost identical both times and the same as in analyses before and after these two batches.

Statistical methods

The food data are presented as arithmetic means (±SD) and the lignan and phytoestrogen results as arithmetic means (±SD) and geometric means. Geometric means were used when necessary because of skewness of the distribution of the results. The statistical analyses were carried out by using the *StatView* program for Macintosh (Abacus Concepts, Berkeley, CA). The degree of univariate associations between two variables were estimated as Pearson's correlation coefficients (r). The pairs of

TABLE 1
Intake of various food stuffs by the Japanese women and men consuming a traditional Japanese diet*

Nutrient	Women (n = 10)	Men (n = 9)
	g/d	
Rice	578.5 ± 222.5	764.7 ± 240.3
Wheat	59.5 ± 46.0	139.0 ± 113.6
Potato	62.6 ± 30.2	55.2 ± 34.6
Sugar	8.1 ± 7.0	8.1 ± 7.4
Fats	13.1 ± 7.6	12.7 ± 6.9
Pulses and beans	56.5 ± 36.0	40.9 ± 32.0
Fruit	228.2 ± 111.9	146.9 ± 114.0
Green and yellow vegetables	60.6 ± 33.3	55.7 ± 35.2
Other vegetables	139.3 ± 69.3	130.9 ± 77.2
Pickles	32.9 ± 24.9	23.2 ± 21.2
Algae	1.8 ± 2.0	0.7 ± 0.7
Fish	98.7 ± 46.6	113.6 ± 56.5
Meat	37.0 ± 30.1	73.6 ± 58.4
Eggs	38.4 ± 16.6	57.4 ± 30.6
Milk	112.7 ± 131.0	90.9 ± 90.2
Beer	5.1 ± 16.1	454.6 ± 647.1

* $\bar{x}$ ± SD.

adjusted group means for the two groups studied (women and men) were compared by nonpaired t test.

Results

The intake of various types of food are shown in Table 1, and Table 2 shows the results of the calculations with regard to energy;

TABLE 2
Energy intake, intake of various nutrients, and some ratios in the two study groups*

Nutrient	Women (n = 10)	Men (n = 9)
Energy		
(MJ/d)	8.29 ± 1.64	10.79 ± 3.48
(kcal/d)	1973 ± 391	2569 ± 829
Animal protein (g/d)	35.3 ± 13.9	47.8 ± 18.9
Vegetable protein (g/d)	38.2 ± 10.1	45.1 ± 10.6
Total protein (g/d)	73.6 ± 12.2	93.0 ± 28.4
Carbohydrates (g/d)	311.4 ± 77.0	383.3 ± 100.6
Total fat (g/d)	44.4 ± 14.4	51.0 ± 25.9
Total fiber (g/d)	16.9 ± 4.9	15.3 ± 6.0
Animal protein (%)†	47.2 ± 15.9	49.8 ± 7.9
Proteins (%)‡	15.2 ± 2.1	14.6 ± 1.5
Carbohydrates (%)‡	64.6 ± 6.8	68.2 ± 5.1
Fats (%)‡	20.3 ± 5.5	17.2 ± 4.9
Fat (g/kg body wt)	0.86 ± 0.31	0.85 ± 0.37
Fiber		
(mg/J)	2.1 ± 0.7	1.5 ± 0.7
(g/1000 kcal)	8.8 ± 3.0	6.4 ± 3.0
Fiber (g/kg body wt)	0.33 ± 0.10	0.26 ± 0.09
Fat-fiber ratio	2.5 ± 0.9	2.4 ± 0.9

* $\bar{x}$ ± SD.
† Percent of total protein.
‡ Percent of energy.

TABLE 3
Dietary intake of soy products by the two groups studied*

Soy product	Women (n = 10)	Men (n = 9)
	g/d	
Tofu (soybean curd)	25.0 ± 22.9	18.7 ± 28.8
Miso (bean paste)	12.5 ± 6.2	8.5 ± 6.4
Aburaage (fried thin tofu)	2.6 ± 3.6	3.7 ± 4.2
Atuage (fried thick tofu)	4.0 ± 12.7	0.8 ± 2.3
Koridofu (dried soybean curd)	0.37 ± 0.78	0.07 ± 0.2
Fermented soybeans	2.4 ± 4.5	0.9 ± 2.8
Boiled beans	7.7 ± 17.8	6.5 ± 7.8
Soy sauce	22.9 ± 6.1	19.2 ± 4.7
Soy products (sauce excluded)	54.4 ± 34.3	39.2 ± 36.4

* $\bar{x}$ ± SD.

animal and vegetable protein; total proteins, carbohydrates, fats, and fiber; percentage animal protein and percentage protein; and carbohydrate and fat as percent of total calories. Furthermore, we calculated the fat intake per kilogram body weight, fiber intake per J (per 1000 kcal), and the fat-fiber ratio (Table 2). The diet was a low-fat (fat 17.2% and 20.3% of total calories for men and women, respectively), low-animal-protein diet with moderate amounts of fiber and a low fat-fiber ratio, which is typical for the traditional Japanese diet (37).

Table 3 shows the dietary intake of soy products, which were expected to be the most important source of precursors for the urinary isoflavonoids (3).

Table 4 shows the mean excretion values for the two lignans and three isoflavonoid phytoestrogens. The results show a relatively low excretion of enterolactone, a normal excretion for enterodiol, and a very high excretion of isoflavonoid phytoestrogens. The individual results showed large variation, particularly for equol (from 0 to 10.95 μmol/d). For comparison note that the geometric mean values in young omnivorous women living in Helsinki and in Boston for enterolactone, enterodiol, daidzein, equol, and *O*-desmethyl-angolensin were 2.46, 0.20, 0.22, 0.10, 0.03, and 2.05, 0.28, 0.32, 0.07, and 0.03 μmol/d, respectively (2).

TABLE 4
Urinary excretion of lignans and isoflavonoid phytoestrogens in Japanese women and men consuming traditional Japanese diet*

Compound	Women (n = 10)	Men (n = 9)
	μmol/d	
Enterolactone	1.4 ± 1.4 (0.89)	1.1 ± 0.7 (0.89)
Enterodiol	0.7 ± 1.3 (0.41)	0.4 ± 0.3 (0.22)
Total lignans	2.1 ± 2.6 (1.38)	1.5 ± 0.9 (1.13)
Daidzein	2.6 ± 4.0 (2.55)	2.2 ± 2.0 (1.45)
Equol	2.6 ± 4.0 (0.56)	3.0 ± 4.6 (0.54)
O-desmethylangolensin	0.7 ± 0.6 (0.51)	0.2 ± 0.3 (0.11)
Total isoflavonoids	6.9 ± 6.8 (4.73)	3.9 ± 3.3 (2.57)
Total diphenols	9.1 ± 9.3 (6.7)	5.4 ± 4.0 (4.1)

* $\bar{x}$ ± SD (geometric $\bar{x}$).

Table 5 presents a correlation matrix of various food components and urinary excretion of lignans and isoflavonoids in the total material of 19 subjects for whom both food and phytoestrogen data were available.

Discussion

In a previous study of oriental immigrant women from southeast Asia residing in Hawaii (38), the diet was similar to that consumed by the men and women in the rural village in Japan. In the present study the women had a greater energy intake (an additional ~2.1 MJ/d, or 500 kcal/d), which may be due to a physically more active life. However, the percentage intake of calories as fat and the dietary fiber and fat-fiber ratio were very similar to the corresponding values in the previous study. Except for the energy intake the values are very different from those seen in Western societies where the fiber intake is similar but the fat-fiber ratio is much higher. Women living in the Boston area had a fat-fiber ratio of 7.7 for the premenopausal women and 4.6 for the postmenopausal women compared with 2.5 for the women in the present study (39).

With regard to protein intake, expressed as g/d and as percentage of calories, the mean values in the present study were similar and slightly lower, respectively, than those of the immigrants from southwest Asia (38).

Our results are in good agreement with those from an earlier study of 300 female agricultural workers from 18 regions in Japan (37) except for dietary fiber intake, which was much lower (between 5 and 6 g/d) in the women in the earlier study (which may represent crude fiber intake). However, according to the national nutrition survey in Japan, the dietary fiber intake was 22.8 g/d in 1951 and decreased year by year to 17.4 g/d in 1985. These figures are in better agreement with our results obtained in 1985, which show a mean dietary fiber intake in the whole group of ~16 g/d. This latter value is also in good agreement with the value of 13 g/d for nonstarch polysaccharides found by analyses of the Japanese diet in another study (40). On the basis of these investigations and the present investigation, it may be concluded that the amount of dietary fiber in a traditional oriental diet is comparable with that in many Western societies (38–40). We may also conclude that the diet of our subjects was typical for a rural area, where the people to a large extent consume their own products and have a traditional Japanese diet.

The urinary excretion of Enl was, with few exceptions, low in both men and women (Tables 4 and 1A) and was the same as found for the postmenopausal breast-cancer patients in Boston (20). We found a weak correlation between intake of green and yellow vegetables and excretion of Enl and total lignans (Table 5) but no correlation with rice intake. Because these subjects consumed large amounts of rice, it seems justified to conclude that refined rice contains very low amounts, if any, of lignan precursors. There was a better correlation with the intake of soybeans, which thus also may be a source of Enl precursors (Table 5). It is known that soy sauce contains coniferyl alcohol the building block for lignans and lignin (41). The excretion of the lignan End was also found to be associated with the intake of beans and pulses and soy products in general (Table 5).

The excretion of the isoflavonoid phytoestrogens is very high in these Japanese men and women compared with values obtained in women living in Boston (2, 20) and in the Helsinki area (2, 18). The Japanese women in the present study excrete 10 times more Da and 20–30 times more Eq and *O*-Dma than did omnivorous and lactovegetarian women living in the abovementioned two cities. Of the 19 subjects, 47% and 89% excrete micromole amounts of Eq and Da per day, respectively, a phenomenon very rarely seen in subjects consuming a Western diet but seen in subjects consuming a macrobiotic diet (2). The values in an additional study group of nine subjects, including three children (*see* Appendix A), were not significantly different from those in the two main groups (Tables 4 and 1A); they were in fact surprisingly identical. The excretion of matairesinol, the precursor lignan for enterodiol, was very low, but genistein excretion was very high. Genistein is the center of interest in many laboratories because of its very interesting antiproliferative and

TABLE 5
Correlation matrix of various food components and urinary excretion of ligans and isoflavonoids in the whole material (n = 19)

Nutrient	Enterolactone	Enterodiol	Total lignans	Daidzein	Equol	*O*-Desmethylangolensin	Total isoflavonoids	Total diphenols
Green and yellow vegetables	0.525*		0.460*					
Pulses and beans		0.541*	0.492*	0.679†	0.737†	0.617†	0.668†	0.693†
Algae				0.561*			0.450‡	0.430‡
Total fat					0.584†			
Percent fat calories								
Fat-fiber ratio					0.469*			
Meat					0.507*			
Soy products (not sauce)		0.481*		0.583†	0.507*			
Boiled soybeans	0.758§	0.892§	0.849§	0.632†	0.746§	0.601†	0.585†	0.588†
					0.693§		0.757§	0.801§

* $P < 0.05$.
† $P < 0.01$.
‡ $0.05 < P < 0.10$.
§ $P < 0.001$.

antimitogenic effects (*see* below); genistein showed the highest concentration of all phytoestrogens in urine in these nine subjects. The mean value was almost 6 μmol/d and a value as high as 15.5 μmol/d was observed. Also in this smaller group most variation in the excretion values was found for Eq (from 0.01 to 9.16 μmol/d). In 21.4% of all subjects, equol excretion did not significantly differ from zero: this group included two of the three children; the mother of these two children did not excrete equol in significant amounts.

The low excretion of Enl in the Japanese subjects compared, eg, with Finnish women (2), is most likely due to low intake of grain (whole-grain) products such as bread (2, 17, 18, 42, 43). The precursors of the mammalian lignans seem to be located in the aleuronic layer of the grain close to the fiber (15) but definite evidence for this location has not yet been obtained. The mean End values are similar to those observed in lactovegetarian American and Finnish women and higher than in the omnivorous women from the same countries (2, 20). It is likely that the majority of the lignans in these Japanese subjects is derived from nongrain plant products (pulses and beans), as suggested by the correlations found in Table 5.

Eq excretion correlated positively with the intake of total fat ($P < 0.01$), fat-fiber ratio ($P < 0.05$), and meat ($P < 0.05$) and deviated in this aspect from all the other isoflavonoids. Some subjects are not able to produce Eq at all, as also shown previously for nonJapanese subjects (44). It is possible that those consuming more fat and meat have an intestinal flora more capable of producing Eq from Da, known to occur in large amounts in soybeans (45). Algae may also be a source of isoflavonoids because a positive correlation was found with Da ($r = 0.56$; $P < 0.05$) and total isoflavonoids ($r = 0.45$; $0.05 < P < 0.10$, NS). Algae were suggested to contain factors protective against breast cancer (46).

Lignans and bioflavonoids are candidates for a role as cancer-protective agents (2, 14–16) and as steroid competitors for various enzymes (47). Enl inhibits the aromatase enzyme and competes with the natural substrate androstenedione for the binding site on the cytochrome P450 enzyme (H Adlercreutz, C Bannwart, LE Vickery, et al, unpublished observations, 1985). Phytoestrogens and lignans (48; H Adlercreutz, Y Mousavi, J Clark, et al, unpublished observation, 1987) show interaction with estrogen receptors and flavonoids have antiproliferative effects on the human-breast-carcinoma cell line ZR-75-1 (49). Genistein is a very specific inhibitor of the tyrosine-specific protein kinases (50–55) and platelet-activating-factor–stimulated platelet aggregation, phospholipase C, and tyrosine kinase activity (56). Tyrosine kinase is an important mediator of the effects of some biologically important growth factors such as epidermal growth factor, insulin, platelet-derived growth factor, and insulin-like growth factor on cells. The flavonoids and lignans bind to the type II estrogen-binding sites (15, 57), now also called the bioflavonoid receptor (47, 58), and may in this way regulate by inhibition cell growth and proliferation of hormone-dependent cancers (58). Enzymes metabolizing bioflavonoids and steroids show structurally close similarity (47), indicating that they have the same origin. Furthermore, the isoflavonoid coumestrol complements, as does estradiol, the topography of spaces between base pairs in unwound DNA and simultaneously hydrogen-bond phosphate moieties on opposite strands (59).

One of the most important biological effects of the lignans and isoflavonoids seems to be their stimulation of SHBG synthesis in the liver (2, 14, 16–18). A high SHBG concentration leads to decreased metabolic clearance rate for the sex hormones and lower biological activity. However, Japanese and British women were found to have the same SHBG total-binding capacity, even though Japanese women bound relatively more estradiol to SHBG. This was suggested to be a result of lower affinity of albumin for estradiol in these women (60). It is possible that the phytoestrogens in the high amounts occurring in Japanese women could compete with estradiol for the albumin-binding sites and in this way lead to relatively more binding to SHBG.

SHBG concentrations tend to be lower in breast-cancer patients, particularly in postmenopausal women, and this seems at least partly to be due to diet (15). SHBG-binding capacity was significantly smaller in postmenopausal but not in premenopausal Japanese subjects with breast cancer compared with Japanese control subjects (61), agreeing with our own more recent results in American postmenopausal (43) women. Finnish premenopausal women with breast cancer did not differ in this respect from omnivorous control subjects but they had lower SHBG than did lactovegetarian women (18). Diet seems to be a much more important risk factor for postmenopausal than for premenopausal breast cancer (15). Miso (Japanese soybean paste) (62) or powdered soybean chips (63) (both before and after denaturation of the protease inhibitors) showed a tendency to decrease mammary-tumor formation and growth rate in rat breast-cancer models and soybean diet also reduced breast-tumor incidence in irradiated rats (64). This agrees with the slower average growth rate of postmenopausal breast cancers in Japanese compared with caucasian women in Hawaii (65).

The high concentration of phytoestrogens in the urine of Japanese men could be protective with regard to prostate cancer. Both lignans and isoflavonoids have estrogenic effects in numerous biological systems and may, because of this property, inhibit development of prostatic cancer. It is well known that in Japan and some other Asian countries, despite the same incidence of latent small or noninfiltrative prostatic carcinomas as in Western societies, the mortality is low (25–27). The high exogenous phytoestrogen concentrations could inhibit the growth of the latent carcinomas, postponing their development and making it more likely that the subjects die from some other disease (theory proposed in 1985) (66). Furthermore, the inhibitory effect of genistein on tyrosine-specific protein kinases of certain growth-factor receptors could play an important role. Decreased risk of prostate cancer is seen in Seventh-day Adventist men (67) consuming much beans, lentils, and peas and some dried fruits (rich sources of bioflavonoids) and in men of Japanese ancestry in Hawaii consuming much rice (mainly starch, which has some fiber-like effects in the gut) and tofu (68), supporting the view that these compounds are protective. Recently, Santti's group in Turku, Finland, in a collaborative study with us, observed that dietary soy prevented the development of precancerous changes in a neonatally estrogenized mouse used as a model for prostatic cancer (69), showing that dietary factors may already be important in the fetal and neonatal periods. This study and our observation of high phytoestrogen excretion in urine of children is important because they suggest that these compounds may change the endocrine milieu at the cellular level both in the neonatal period and in prepubertal and adolescent children. Thus, the results cited above and discussed more

extensively elsewhere (14, 15) speak for a role of the diphenols as cancer-protective substances.

It is concluded that Japanese subjects excrete very large amounts of isoflavonoids in urine, mainly genistein, daidzein, and equol, and that the lignan excretion is low. The high excretion of isoflavonoids in urine is related to the intake of soy products in the traditional Japanese diet.

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APPENDIX A

Additional experiments with a modification of the method

The method used in this study was modified further by including the determination of the plant lignan matairesinol [(3R-*trans*)-dihydro-3,4-*bis*[(4-hydroxy-3-methoxy-phenyl)methyl]-2(3H)-furanone)] (intraassay CV = 15.2% and interassay CV = 13.9%) and the isoflavonoid genistein (4′,5,7-trihydroxyisoflavane) (intraassay CV = 4.5% and interassay CV = 11.6%) in the assay (1). Because further samples from the present study were not available and because of the recent great interest in genistein we used this new assay in nine other Japanese subjects (three men, three women, and three children) living in Kyoto and consuming a traditional Japanese diet before and during the 24-h urine collection.

TABLE 1A
Urinary excretion of lignans and isoflavonoid phytoestrogens (μmol/d) in nine Japanese subjects (six adults, three children) living in Kyoto and consuming traditional Japanese diet during the urine collection period

Subject, sex, age	Matairesinol	Enterolactone	Enterodiol	Total lignans	Daidzein	Equol	*O*-Desmethylangolensin	Genistein	Total isoflavonoids	Total diphenols
1, M, 41 y	0.010	0.05	0.09	0.15	5.25	6.15	0.12	15.52	27.04	27.20
2, F, 33 y	0.003	2.44	0.15	2.59	3.11	0.01	0.98	4.48	8.58	11.17
3, M, 7 y	0.003	0.07	0.09	0.16	3.23	0.01	0.06	5.66	8.97	9.13
4, M, 6 y	0.006	2.24	0.68	2.93	2.15	0.85	0.51	3.41	6.93	9.85
5, M, 8 y	0.007	0.04	3.39	3.43	3.02	0.02	0.81	4.80	8.64	12.07
6, F, 42 y	0.006	3.25	0.25	3.50	2.20	0.16	1.17	3.55	7.07	10.58
7, M, 38 y	0.012	0.70	0.25	0.96	1.60	0.07	0.40	4.93	6.99	7.95
8, M, 26 y	0.019	1.94	0.18	2.13	3.38	9.16	0.23	7.99	20.76	22.89
9, F, 30 y	0.005	0.62	0.25	0.88	1.25	3.28	0.21	1.85	6.60	7.47
$\bar{x}$	0.010	1.26	0.59	1.86	2.8	2.19	0.50	5.80	11.29	13.15
Geometric $\bar{x}$	0.010	0.50	0.27	1.17	2.58	0.25	0.35	4.91	9.81	11.89

Table 1A shows the individual urinary lignan and isoflavonoid excretion in the additional three men, three women, and three children studied by the new modified procedure, including the results of assays for matairesinol and genistein.

Reference

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BIOMEDICAL AND ENVIRONMENTAL MASS SPECTROMETRY, VOL. 18, 973–977 (1989)

The Use of Thermospray Liquid Chromatography/ Tandem Mass Spectrometry for the Class Identification and Structural Verification of Phytoestrogens in Soy Protein Preparations

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The thermospray mass spectra of the phytoestrogens have intense protonated molecular ions but contain few or no ions indicative of structure. Tandem mass spectrometry (MS/MS) was used to obtain daughter ion spectra containing ions unique to the different structural characteristics of each phytoestrogen subclass and was used both to confirm identification and propose structures for unknowns. In addition to unique daughter ion spectra, MS/MS was used as a class identifier to detect phytoestrogens through the neutral loss of 56 (due to consecutive losses of CO) that is common to all members of this family. Several sources of soy protein were investigated to confirm the presence or absence of phytoestrogens. In one preparation investigated, daidzein and genistein were detected as well as an unknown phytoestrogen of the Biochanin A subclass. This unknown has been tentatively identified as 6,7-dihydroxy-4'-methoxyisoflavone using its daughter ion spectrum.

INTRODUCTION

The ubiquitous occurrence in plants of compounds possessing estrogenic activity[1,2] has led to extensive studies of their effects in animals following their ingestion.[3] Deleterious effects of dietary estrogens upon reproduction have been reported for several animal species, including sheep,[4] California quail,[5] rats[6] and exotic cats,[7] and attention has recently focused on the potential role of dietary estrogens in man.[8–10]

Two main classes of non-steroidal phytoestrogens occur in plants—the isoflavones and coumestans—and both groups are heterocyclic phenols having close three-dimensional structural similarity to the naturally occurring mammalian estrogenic steroids[3] and bind to uterine estrogen receptors[11,12] which induce estrogenic biological responses.

Suitable methods are therefore required for the detection of this class of compounds and a technique utilizing liquid chromatography with ultraviolet (UV), electrochemical detection and thermospray ionization/mass spectrometry was recently developed.[13] The latter procedure, however, yields mass spectra characterized by intense protonated molecular ions with no structural information.[13] We therefore extended these studies and now report on the use of a thermospray ionization liquid chromatography/tandem mass spectrometry (LC/MS/MS) technique for these compounds that affords greatly increased specificity and allows the screening for compounds bearing commonality in structure. The method has been applied to the detection of phytoestrogens in a variety of diets and biological fluids.

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EXPERIMENTAL

Materials and reagents

Biochanin A, genistein, daidzein and formononetin were obtained from K&K Rare and Fine Chemicals (Plainview, New York, USA) and coumestrol from Kodak (Rochester, New York, USA), Equol was obtained from the MRC Steroid Reference Collection (curator Prof. D. N. Kirk, Queen Mary College, London University). Burdick & Jackson high-purity solvents (Muskegon, Michigan, USA) were used for chromatography and sample preparation. The enzyme preparation β-glucosidase was obtained from Sigma (St Louis, Missouri, USA).

Sample preparation

Samples of soy protein (5 g) were homogenized in 80% ethanol (250 ml) and refluxed for 2 h to extract isoflavones, their conjugates and related compounds. The organic extracts were cooled, centrifuged and the supernatant was removed. The ethanol was evaporated in a rotary evaporator and lipids were extracted from the remaining aqueous extract by partitioning twice into 4 vols of hexane. The aqueous extract was taken to dryness and isoflavone conjugates were hydrolysed with a β-glucosidase prepared in 0.1 M acetate buffer overnight. The hydrolysate was passed through a cartridge of reversed-phase octadecylsilane-bonded silica (Bond-

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Elut C_{18}; Analytichem, Harbor City, California, USA) to extract all isoflavones, and after washing the cartridge with water the isoflavones were recovered by elution with 5 ml methanol. The methanol extract was taken to dryness under nitrogen on a 65 °C heating block, and the residue was reconstituted prior to assay.

LC/MS/MS

Mass spectrometry was performed on a Finnigan MAT TSQ-46 (Finnigan MAT, San Jose, California, USA) triple-stage quadrupole. Daughter ion spectra of reference compounds were obtained using the standard Finnigan ion source operating in the chemical ionization (CI) mode with ammonia as the reagent gas. Source temperature was 120 °C with an indicated source pressure of 0.5 mtorr. Standard samples were introduced using the direct exposure probe. For combined LC/MS/MS the instrument was fitted with a Vestec 701 thermospray ion source and controller (Vestec, Houston, Texas, USA). All the MS/MS experiments were run using argon as the collision gas at an indicated collision cell pressure of 1.9–2.0 mtorr. Collision energy was set at −30.0 eV (lab reference). The various daughter ion and neutral loss spectra were acquired under data system control during the entire chromatographic run.

The thermospray interface was optimized for the separation conditions of the phytoestrogens. The ion source (block) temperature and tip heater were set to 250 °C. The vaporizer temperature was set to 148 °C, which resulted in a tip vaporizer temperature of 182 °C and a vapor temperature of 240 °C. A Waters 600-MS high-performance liquid chromatography (HPLC) system (Waters, Milford, Massachusetts, USA) was used. In-line UV detection at 260 nm[13] was obtained using a high-pressure cell in a Waters 490-MS detector, and UV data were collected using the mass spectrometer data system. The column (25 cm × 4.6 mm i.d.) was Hypersil ODS, 5 μm. The mobile phase was methanol:0.1 M ammonium acetate, pH 4.6 (60:40 v/v) and the flow rate was 1.2 ml min^{-1}.

RESULTS AND DISCUSSION

In order to obtain an understanding of the collisional activation decomposition (CAD) of the phytoestrogens, standards of daidzein, genistein, coumestrol, formononetin and Biochanin A were run by direct probe using ammonia CI. A summary of the MS/MS fragmentation of these standards is found in Table 1. These compounds can be grouped into three classes. The only two common ions for all the phytoestrogens are the protonated molecular ions and the ion resulting from the loss of 56 daltons from the protonated molecular ion. The loss of two carbon monoxide groups accounts for this neutral loss of 56 daltons.

Each class of phytoestrogens has unique ions resulting from neutral loss or radical loss which serve as class identifiers. Figure 1 presents the CAD fragmentation pathways of the phytoestrogen classes.

Daidzein and genistein constitute class (a), which is characterized by a unique neutral loss of water from the protonated molecular ion. Other daughter ions arise from a retro–Diels–Alder rearrangement (RDAR) from the same ion.

Table 1. Summary of MS/MS fragments for three classes of phytoestrogens

Fragment	Class (a) Daidzein / Genistein	Class (b) Formononetin / Biochanin A	Class (c) Coumestrol
$[M+H]^+$	X	X	X
$-{}^{\cdot}CH_3$[c]		X	
$-CH_4$		X	
$-H_2O$[b]	X		
$-CO$	X		X
$-CH_3OH$[c]		X	
$-{}^{\cdot}CH_3 - CO$		X	
$-CH_4 - CO$		X	
$-CO_2$[d]			X
$-2CO$[a]	X	X	X
$-CO - CO_2$			
$-4CO$			X

[a] Class identifier for entire phytoestrogen family (neutral loss 56).
[b] Subclass identifier for daidzein, genistein and similar compounds (neutral loss 18).
[c] Subclass identifier for formononetin, Biochanin A and similar compounds (neutral loss 15 and 32).
[d] Subclass identifier for coumestrol (neutral loss 44).

Formononetin and Biochanin A, which make up class (b) have a number of unique daughter ions. Formononetin and Biochanin A show losses of a methyl radical, methane and methanol. In addition, losses from the protonated molecular ion include a methyl radical followed by loss of carbon monoxide, methane with subsequent loss of carbon monoxide, and an RDAR.

Coumestrol does not show a unique neutral loss ion, but the ion resulting from the loss of 44 daltons (carbon dioxide) is indicative of the coumestrol class. Formononetin and Biochanin A also have a daughter ion resulting from the loss of 44 daltons, but this daughter ion results from first the loss of methane (16 daltons) followed by the loss of carbon monoxide (28 daltons).

One other non-steroidal estrogen, equol, a compound formed by bacterial modification of other phytoestrogens, was analyzed by MS/MS. Equol, 3,4-dihydro-3(4-hydroxyphenyl)-2*H*-1-benzopyran-7-ol, does not have the 4-oxo group on the benzopyran group and therefore did not show the common neutral loss of 56 daltons. Equol has a protonated molecular ion at *m/z* 243 and the following daughter ions: *m/z* 149, M + H − phenol; *m/z* 133, M + H − 3-hydroxyphenol; *m/z* 123, M + H − 4-hydroxystyrene; *m/z* 107, 4-hydroxybenzyl ion; and *m/z* 105, *m/z* 133 − carbon monoxide. Although equol would not be detected with the 56 daltons neutral loss scan, a daughter ion scan of *m/z* 243 would confirm the presence or absence of equol.

Based on this information, an MS/MS experiment was designed to acquire daughter ion spectra of three ions to confirm the presence or absence of four phytoestrogens: *m/z* 255 (daidzein), *m/z* 271 (genistein) and *m/z* 269 (coumestrol and formononetin). Biochanin A was not expected to be present based on preliminary HPLC analysis but a neutral loss of 56 scan was run to

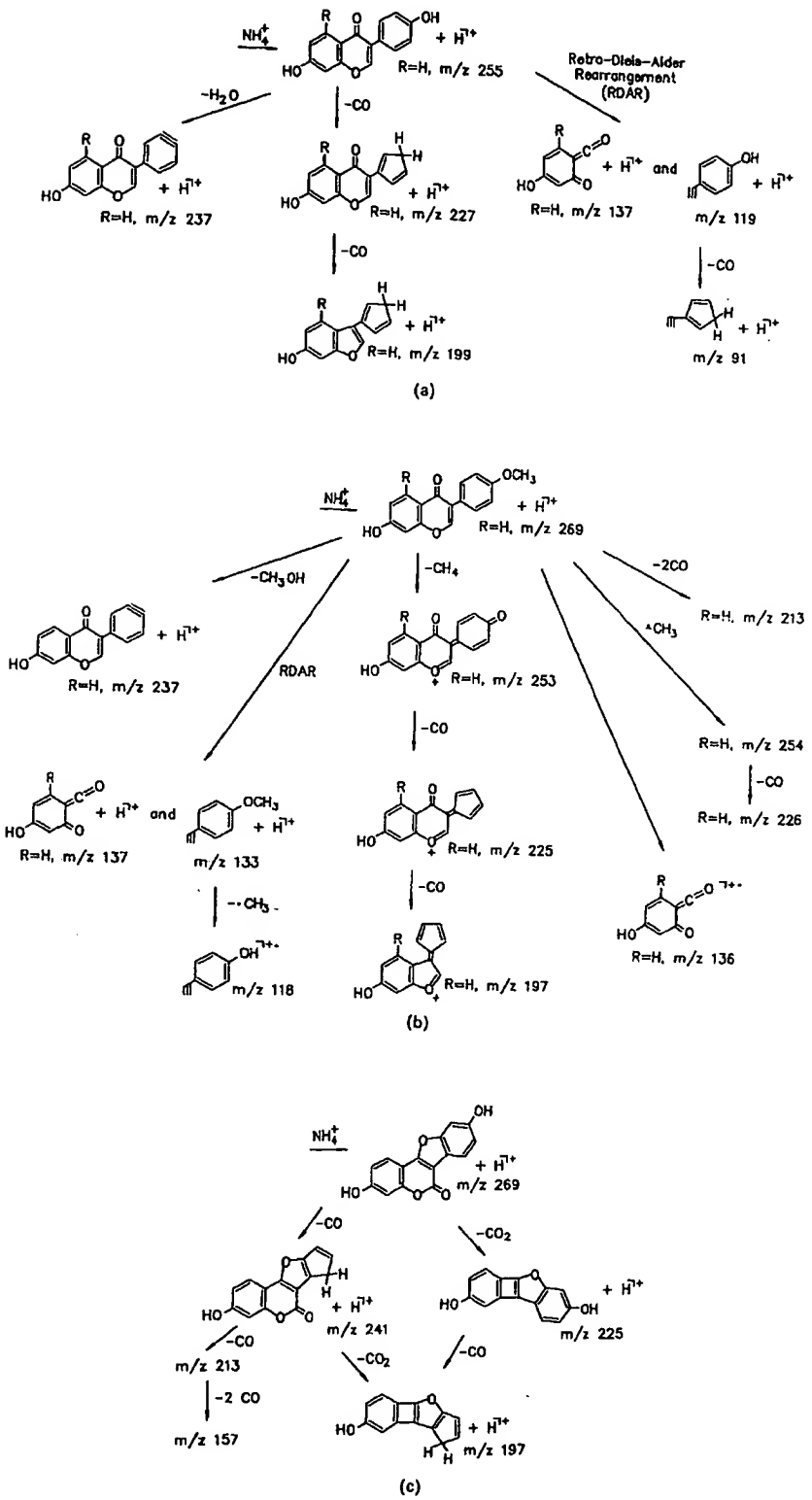

Figure 1. Proposed CAD fragmentation pathways of the phytoestrogen classes. (a) Daidzein (R = H) and genistein (R = OH). (b) Formononetin (R = H) and Biochanin A (R = OH). (c) Coumestrol.

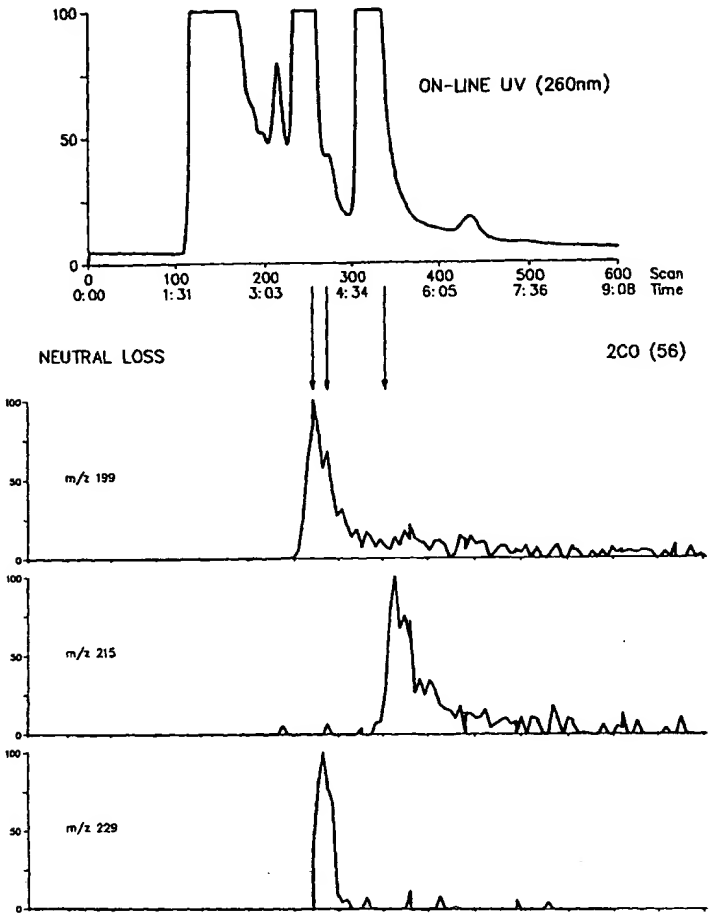

Figure 2. Old-Nebraska Cheetah diet; LC/MS/MS experiments.

detect this or any other phytoestrogen. This cycle of three daughter ion scans and one neutral loss was repeated throughout the entire HPLC run.

Methods for the analysis of phytoestrogens in soy protein products have generally relied upon the use of HPLC with UV detection.[14–20] In our experience confidence in peak identification is the major drawback that can be overcome by the use of LC/MS techniques. In this study over 20 different preparations of vegetable and vegetable-containing diets were screened. In several cases, false positives obtained by UV detection alone were shown not to contain phytoestrogens. In the case of an old formulation of a commercially produced Cheetah diet (Nebraska Feline diet, Lincoln, Nebraska) that contained soy protein, the analysis by UV indicated the presence of daidzein and genistein.[7] When this sample was run by LC/MS/MS the UV chromatogram and neutral loss daughter ion mass chromatograms shown in Fig. 2 were obtained. In addition to the expected daughter ions at *m/z* 199 and *m/z* 215, another ion at *m/z* 229 was observed. With the neutral loss of 56 daltons, this unknown phytoestrogen would have a protonated molecular ion at *m/z* 285.

The presence of daidzein and genistein was confirmed by their daughter ion spectra shown in Fig. 3(a) and (b). The daughter ion spectrum of *m/z* 285 for the unknown phytoestrogen is shown in Fig. 3(c). This unknown phytoestrogen had the same molecular weight as Biochanin A, but the daughter ion spectrum and the chromatographic retention times were different for these two phytoestrogens. Based on its daughter ion spectrum, this unknown has been tentatively identified as 6,7-dihydroxy-4'-methoxyisoflavone. Even though this unknown phytoestrogen co-eluted with daidzein, the use of a neutral loss scan as a class identifier allowed the detection of another component in the mixture. By rerunning the sample and collecting daughter ion spectra of the suspected protonated molecular ion, a tentative identification could be made.

This unknown phytoestrogen has the same protonated molecular ion (*m/z* 285) as Biochanin A and a comparable daughter ion profile, but the intensities of the daughter ions for the two compounds are different. The following daughter ions were observed for this phytoestrogen tentatively identified as 6,7-dihydroxy-4'-methoxyisoflavone: *m/z* 270, M + H − methyl radical;

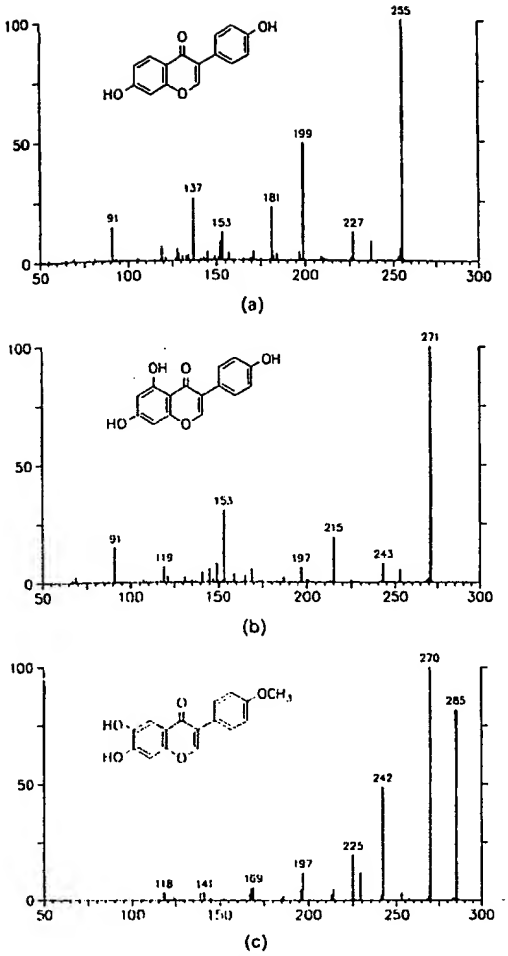

Figure 3. (a) MS/MS daughter ion mass spectrum of daidzein (*m/z* 255) from Old-Nebraska Cheetah diet. (b) MS/MS daughter ion mass spectrum of genistein (*m/z* 271) from Old-Nebraska Cheetah diet. (c) MS/MS daughter ion mass spectrum of *m/z* 285 (tentatively identified as 6,7-dihydroxy-4'-methoxyisoflavone) from Old-Nebraska Cheetah diet.

m/z 257, M + H − carbon monoxide; *m/z* 253, M + H − methanol; *m/z* 242, M + H − methyl radical − carbon monoxide; *m/z* 229, M + H − 2 carbon monoxides; *m/z* 225, M + H − methanol − carbon monoxide; *m/z* 214, M + H − methyl radical − 2 carbon monoxides; and *m/z* 197, M + H − methanol − 2 carbon monoxides.

The daughter ions resulting from the losses of 15, 32 and 43 daltons are consistent with the 4'-methoxyphenyl group of the formononetin and Biochanin A subclass. The low-intensity daughter ions at *m/z* 152 and 124, from RDAR, place the hydroxyl on the phenyl group of benzopyran-4'-one.

These results demonstrate the utility of thermospray LC/MS/MS for the class identification and structural assignments of phytoestrogens in dietary preparations. Furthermore, since these non-steroidal compounds are also excreted in the urine of subjects ingesting soy protein products[7] the technique could be extended to permit their detection in urine extracts.

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SHORT PAPERS

OESTROGENIC RESPONSE OF THE CD-1 MOUSE TO THE SOYA-BEAN ISOFLAVONES GENISTEIN, GENISTIN AND DAIDZIN

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Abstract—The soya-bean isoflavones genistein and daidzein have been reported to exhibit oestrogenic activity in mice, but there is a significant difference in the response of different mouse strains to these compounds. The CD-1 mouse was found not to respond to these oestrogenic compounds from soya-beans or to diethystilboestrol at levels previously reported to be effective in other strains.

Introduction

Many naturally occurring compounds that exhibit oestrogenic activity are widely distributed in both plants and animals. Among the compounds that exhibit such activity are the soya-bean isoflavones, genistein and daidzein. The glucosides of genistein and daidzein (genistin and daidzin) are the major forms of isoflavones in soya beans. These compounds have not been assayed for their biological potency as oestrogens, but oestrogens in pasture legumes have been assayed by various workers using mice, guinea-pigs and sheep (Bennet, Morley & Axelsen, 1967; Davies & Bennet, 1962; Morley, Bennet, Braden *et al.* 1968). These studies have indicated that phyto-estrogens differ in potency among animal species. Most of the information available on the effects of the oestrogenic isoflavones has dealt with the reproductive system of sheep (Newsome & Kitts, 1977; Shutt & Cox, 1972). Little information exists on the possible adverse effects that the phytoestrogens may have in nonruminant animals. Therefore, it is difficult to make projections concerning these oestrogenic effects and the potential hazard that the phyto-estrogens may present to human health.

Several studies of the oestrogenic character of the soya-bean isoflavones have used mice as the experimental animal, an increase in uterine weight in the immature female being considered a positive response. Most reports do not indicate the strain of mouse used (Bickoff, Livingston, Hendrickson & Booth, 1962; Cheng, Story, Yoder *et al.* 1953), but in one report, Fredericks, Kincaid, Bondioli & Wright (1981) indicate a difference between the ICR mouse and the B6D2F1 mouse in their response to coumestrol, an alfalfa phytoestrogen.

We report here bioassay data showing that CD-1 mice do not respond to phytoestrogen levels comparable to those studied in other strains of mice. The levels of diethylstilboestrol (DES) required to produce an increase in uterine weight in the CD-1 strain were also higher than those previously reported to be effective.

Experimental

Animals and diet. Swiss albino CD-1 mice were obtained from Charles River Breeding Laboratory (Portage, MI). Female mice were received, with their dams, when 7, 8, 9 or 10 days old. Upon arrival, dams were caged singly, with their pups. The mice received food and water *ad lib.* The dietary protein source was casein in a pelleted semi-purified diet (AIN Rat and Mouse Diet 76) obtained from United States Biochemical Corp. (Cleveland, OH). The composition (%, w/w) of this diet was: casein high nitrogen (20.0), DL-methionine (0.3), cornstarch (15.0), sucrose (50.0), Fiber-Celufil (5.0), corn oil (5.0), AIN mineral mix (3.5), AIN vitamin mix (1.0) and choline bitartrate (0.2). It was essential to avoid exposure of the young mice to a diet containing soya-bean protein, since diets based on soya-bean protein have been reported to contain substantial quantities of phytoestrogens (Drane, Patterson, Roberts & Saba, 1980; Murphy, Farmakalidis & Johnson, 1982). Mice were weaned when 20 or 21 days old and weanlings weighing between 8 and 11 g were assigned to treatment groups of varying size (see Table 1) according to a completely randomized design.

Chemicals. Diethylstilboestrol, used as a positive control, was obtained from Sigma Chemical Co. (St Louis, MO; lot no. 49C-0092). Genistein was obtained from ICN Rare and Fine Chemicals (Plainview, NY; lot nos 36471-A and 31667-A). Genistin and daidzin were purified from toasted soya-flakes obtained from A. E. Staley Co. (Des Moines, IA) by a method developed in our laboratory (Murphy, 1981) and by a modification of that used by Ohta, Kuwata, Akahori & Watanabe (1979).

Test procedure. The compounds under test were suspended in 5% Tween 80 (Sigma Chemical Co.; lot no. 35B-2480) and were administered to the mice by stomach tube in four daily doses of 0.1 ml suspension/day beginning on day 1 of weaning. The control group was given 0.1 ml 5% Tween 80/day for the 4-day. period. Preliminary experiments showed no adverse or oestrogenic effects due to the carrier.

Table 1. Body-weight and uterine-weight response data for weanling CD-1 mice treated with diehtylstilboestrol, genistein, genistin or daidzin by gastric intubation for 4 days

		Values for mice treated with:						
		5% Tween 80†	Genistein		DES‡		Genistin	Daidzin
Data	Dose...	0.1 ml	6 mg	8 mg	0.6 μg	0.8 μg	12 mg	12 mg
No. of mice/group		56	16	13	15	11	8	8
Mean body weight (g):								
Initial		9.6	9.4	9.4	9.6	10.0	9.9	10.5
Gain (5 days)		3.5	3.4	3.0	2.1	2.1	3.6	3.7
Final		13.1	12.7	12.4	11.7	12.1	13.5	14.2
Uterine weight (mg):								
Mean		25.4	23.8	22.0	77.7*	79.5*	24.0	27.0
Corrected mean		25.1	25.3	23.1	77.9*	77.0*	25.1	28.9*
Uterine weight/body weight ratio × 1000		1.93	1.87	1.77	6.61	6.57	1.77	1.90

DES = Diethylstilboestrol

†Vehicle control.
‡Positive control.
Values are means for the numbers of mice stated. An asterisk denotes a significant difference from the control value at $P < 0.05$.

On day 5 after weaning, mice were weighed and subsequently killed and their uteri were dissected out and weighed immediately without blotting (Bickoff *et al.* 1962). Total doses of the compounds administered were 0.6 and 0.8 μg DES/mouse, 6 and 8 mg genistein/mouse and, for genistin and daidzin, 12 mg/mouse in each case.

Statistical analysis. The data were analysed using a one-way classification analysis of a completely randomized design (SAS Institute Inc., Box 8000, Cary, NC 27511). Analysis of covariance, with uterine weight as the dependent variable of initial and final weights, was carried out to minimize any effects due to weight differences during the experiment. The ratio of uterine weight to final body weight was also calculated. This was another way to adjust for weight effects. The ratio calculated was subjected to statistical analysis. Final weight and weight gain as a function of treatments were subjected to statistical analysis to determine any effects of treatment on the final weight of the animals.

Results and Discussion

Statistical analysis of the data revealed that the initial and final body weights were significant variables. With the use of two different models incorporating initial and final weights of the animals as dependent variables, initial weight was determined to be more significant than final weight for the analysis. The animal weights were therefore adjusted with respect to initial weight only, and were then subjected to statistical analysis. The data are presented in Table 1. 'Corrected mean uterine weights' are the data obtained after adjustment for initial weight differences.

Statistical analysis of both corrected and uncorrected uterine weights indicated no differences between the control group and those given the isoflavones genistein and genistin. A significant increase in uterine weight, compared with the control, was observed for DES and daidzin (corrected value only, in the latter case).

Statistical analysis using the ratio of uterine weight to final body weight indicated no differences between the control group and the mice treated with genistein, daidzin or genistin. Statistical analysis of the data adjusted for both initial and final weight was not carried out. We felt that the observed differences between the control group and the group given daidzin were probably due to final weight effects rather than to an oestrogenic effect, because analysis of the ratio of uterine weight to body weight did not show any significant difference between the control group and the group given daidzin.

Weight gain during the experiment showed no statistically significant differences between the control and treatment groups but was generally lower in the latter groups. Genistein and genistin have previously been reported to depress body-weight gain in male mice (Carter, Matrone & Smart, 1960). Final body weight, although showing no statistically significant differences between treatments, appeared to be an important variable in our experiment. Further experimentation is required to enable conclusions to be drawn on how final weight and weight gain are affected by treatments in this particular type of experiment.

The doses of genistein we used have been reported by other researchers to increase the uterine weight in mice of an unidentified strain (Bickoff *et al.* 1962). The doses of DES used in our experiment were ten times higher than those reported by Bickoff *et al.* (1962) as causing increased uterine weights; doses of DES fed at levels comparable to those used by Bickoff *et al.* (1962) gave no response in the CD-1 mouse (data not shown). The CD-1 mouse was used by other researchers (Leavitt & Wright, 1963 & 1965) in similar experiments (but lasting 10 rather than 5 days) to measure the oestrogenic potency of coumestrol. A total dose of 400 μg coumestrol/mouse was required to produce a 4-mg increase in uterine weight in comparison with the control. The same total dose of coumestrol was reported by Bickoff *et al.* (1962) to increase uterine weight by 30 mg over the control value in mice of an unidentified strain.

The results of our experiment, combined with the previous reports (Leavitt & Wright, 1963 & 1965), indicate that the CD-1 mouse does not respond to doses of oestrogenic compound that have produced

responses in other strains of mice. Therefore, comparisons made between experiments in this strain of mouse and experiments using other strains should be interpreted with caution. The reported differences in response among strains of mouse indicates that standardization of the oestrogen bioassay with respect to mouse strain is required.

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ISOFLAVONE CONTENT OF SOYA-BASED LABORATORY ANIMAL DIETS

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Abstract—The soya phyto-oestrogens (isoflavones)—genistein, daidzein (and their glucosides, genistin and daidzin) and coumestrol—are reported to contribute to the hypocholesterolaemic response to soya-protein foods. Examination of several soya-based diets for laboratory animals has shown the diets to have a highly variable content of isoflavones.

Introduction

The soya isoflavones, genistein, daidzein (and their glucosides, genistin and daidzin) and coumestrol have been recognised for some time as the source of the oestrogenic activity demonstrated in some animal diets by the mouse uterine growth bioassay (Bickoff, Livingston, Hendrickson & Booth, 1962). Assays of oestrogen receptor binding have shown that these isoflavones mimic 17-β-oestradiol, although the binding constants are far less than those of the mammalian oestrogens (Martin, Horwitz, Ryan & McGuire, 1978; Verdeal, Brown, Richardson & Ryan, 1980).

Another reported bioactivity of these isoflavones has been their ability to lower serum cholesterol (Sharma, 1979a,b; Siddiqui & Siddiqui, 1976). In fact, some researchers strongly suggest that the isoflavones in soya are, in part, responsible for the hypocholesterolaemic effect of soya protein compared with animal-protein foods (*Nutrition Reviews*, 1980).

Recently, Drane, Patterson, Roberts & Saba (1980) have reported the extraction of oestrogenically active fractions from various soya-containing laboratory-animal and human foods. We recently reported a method for the quantitative analysis of soya foods for all five forms of the isoflavones (Murphy, 1981) and have now utilized this method to analyse several laboratory-animal diets. We were interested in the isoflavone content of these purified soya-based diets mainly because of their potential use in studies of the ability of plant proteins to lower serum cholesterol. The data show considerable variation in the isoflavone content of these diets.

Experimental

Materials. Soya-based diets for laboratory animals were obtained from several commercial sources. Two (diets 2 and 6) were composed of an enzymatic hydrolysate of soya protein. The other five diets had various protein contents, with little other designation than 'soya protein'. Diets 4 and 5 were from the same supplier and were labelled grades II and I, respectively.

Analysis. Protein was determined according to the AOAC method (Association of Official Analytical Chemists, 1980). The soya isoflavones were determined by a high-performance liquid chromatographic method that we developed recently for soya foods (Murphy, 1981).

Results and Discussion

The diets varied considerably in protein content, as shown in Table 1. Because of this variability, the isoflavone data were expressed in two ways. One was on an "as is" basis, giving the levels in the total diets as received; the other was on a protein basis, so that comparisons between diets could be made. The isoflavone contents of diets 3 and 4 were similar to those of the grade of soya isolates used for human food (Murphy, 1982). The enzymatic hydrolysates (diets 2 and 6) had much lower levels of genistin than did the other diets. We have made similar observations with water-treated soya proteins. When soya beans were germinated, when soya isolate was prepared from analysed varieties of whole soya beans, or when tofu was prepared (by precipitation of water-soluble soya proteins with calcium), the content of the glucoside forms decreased dramatically, but there was no apparent increase in the free forms, genistein and daidzein.

The differences between diets 4 and 5 indicate that the treatment used by the supplier of these diets to change diet 4 (grade II) to diet 5 (grade I) significantly reduced the isoflavone content. This decrease is greater than can be explained by the 23% decrease in protein content.

Coumestrol has been reported to occur as a parts per billion (b = 10^9) component of whole soya beans (Lookhart, Jones & Finney, 1978) and in parts per million in germinated seeds (Lookhart, Finney & Finney, 1980). No coumestrol was detected at the ppb level in these soya-based diets.

The data in Table 1 have also been calculated in terms of the minimum dietary consumption equivalent to 8 mg genistein. This was the minimum dose reported by Bickoff *et al.* (1962) to give an oestrogenic response in mice. The data are also expressed in Table 1 in diethylstilboestrol (DES) equivalents according to Drane *et al.* (1980) and genistein equivalents according to Bickoff *et al.* (1962). These calculations indicate the maximum response that could be expected if all the isoflavone in the diets were in the form of genistein. Few data are available on the bioactivity of the

Table 1. *Isoflavone levels and oestrogenic dose equivalents of commercial soya-based diets for laboratory animals*

Diet no.	Protein concn (%)	Analytical data* (concns in ppm)								Oestrogenic dose equivalents		
		Genistin		Daidzin		Genistein		Daidzein				
		In total diet	On protein basis	In total diet	On protein basis	In total diet	On protein basis	In total diet	On protein basis	Total isoflavone content (ppm genistein equivalents†)	DES equivalent‡ (ppb)	Potentially oestrogenic dietary intakes§ (g)
1	62·01	65	105	16	26	16	26	0	—	93	0·96	86
2	61·60	18	30	14	22	0	—	0	—	28	0·29	283
3	81·56	421	516	73	90	68	83	5	6	546	5·66	15
4	83·17	450	542	67	81	68	82	0	—	566	5·88	14
5	63·64	64	101	18	28	0	—	0	—	77	0·80	103
6	58·31	13	22	0	—	11	19	0	—	24	0·25	332
7	78·81	29	36	0	—	15	19	1	1	44	0·46	181

*Data are the results of triplicate analyses. Recoveries of added isoflavones averaged 78%.
†Total genistein, genistin, daidzin and daidzein (ppm of whole diet); 11 mg daidzein is equivalent to 8 mg genistein.
‡Oestrogenic activity of 8 mg genistein is equivalent to that of 0·083 μg DES.
§Weight of diet provided 8 mg genistein, the minimum dose reported by Bickoff *et al.* (1962) to induce an oestrogenic response in mice.

glucosides, genistin and daidzin, although these forms account for 95–99% of the isoflavone content of whole soya beans (Murphy, 1982; Naim, Gestetner, Zilka *et al.* 1974). Therefore, the data in Table 1 are only a rough estimate of the oestrogenic activity of these diets.

It is not possible at this time to estimate a potential hypocholesterolaemic response to the isoflavones found in soya-based diets. Hypocholesterolaemic activities have been measured in rats for several isoflavones found in lesser-known non-American varieties of legumes (Sharma, 1979a). Daidzein was the only isoflavone found in both these legumes and soya beans. Sharma (1979a) concluded, however, that daidzein had no hypocholesterolaemic activity in rats because not all legumes containing daidzein were able to lower serum cholesterol, The report did not take into account β-sitosterol or the other saponins that would be expected in these legumes and that might be expected to contribute to the hypocholesterolaemic response (Birk & Peri, 1980). Moreover, it is known that the isoflavones are metabolized to different end products in different species (Verdeal & Ryan, 1979). Therefore, it is probable that they elicit different hypocholesterolaemic responses in different species.

Because there is great interest in the cholesterol-lowering ability of soya and other plant-protein foods, we believe it is important that researchers in this area be made aware of the non-protein components of their soya-based diets and the potential effect of these compounds on the interpretation of their data. Much work remains to be done to determine what role, if any, the soya isoflavones play in the hypocholesterolaemic activity of soya-bean protein.

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Estrogens and Related Substances in Plants

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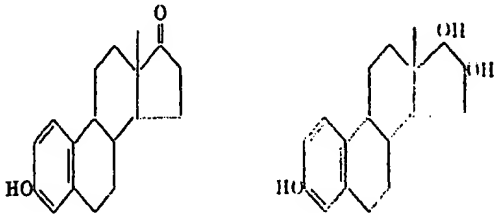
I. Introduction

The occurrence in plants of substances capable of causing estrus in animals was first reported in 1926 (Loewe, 1926; Dohrn *et al.*, 1926; Fellner, 1926) only three years after the development of the Allen-Doisy test (see Emmens, 1950) had made it possible to estimate the effectiveness of different estrogens. Since then many plant extracts have been examined, and a large number of these have been reported to show some estrogenic activity. Indeed, this apparently general occurrence of estrogens caused Dohrn (1927) to doubt the specificity of the Allen-Doisy test for the active "product of the female reproductive glands." Subsequently, it has been clearly demonstrated that the power to cause estrus is by no means confined to substances isolated from female reproductive glands, but is, in fact, shared by a wide variety of substances, many of them obtained only synthetically (*cf.* Masson, 1944; Solmssen, 1945; Jacques, 1949).

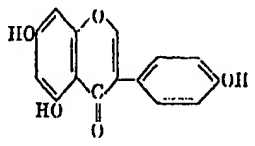
Very few of the positive results obtained in preliminary tests on plants have been followed by larger scale examination and isolation of the plant estrogen, exceptions being the isolation of estrone (I) (Butenandt and Jacobi, 1933) and estriol (II) (Skarzynski, 1933a, b)



I II

These investigations clearly demonstrated the presence in plants of compounds closely resembling the natural animal estrogens. Then Schoeller *et al.* (1940) obtained from *Bulea superba* an estrogen of very great activity which, according to Butenandt (1940) is definitely different from any known member of the follicular hormone group obtained from animals. Its constitution, however, has not yet been elucidated.

The investigation of subterranean clover (*Trifolium subterraneum* L.), undertaken because of the demonstration by Bennetts *et al.* (1946) that the Dwalganup strain of this plant was responsible for serious losses to the sheep-breeding industry in Western Australia, has shown (Bradbury and White, 1951, and references quoted there) that it contains genistein (4′,5,7-trihydroxyisoflavone) (III) and that this compound is weakly estrogenic (*cf.*, Biggers and Curnow, 1954). Subsequently genistein 4′-methyl ether has been isolated from both red and subterranean clovers and shown to be estrogenic (Pope *et al.*, 1953; Beck *et al.*, 1954).



III

Plant estrogens have already been reviewed by Loewe (1933) and very briefly, along with estrogens from animal and mineral sources, by Wehefritz (1936), by Deulofeu (1941a, b), and by Löve and Löve (1945). Since these reviews appeared, however, a number of papers have been

published describing the results of tests with further plant extracts, and the recent work on subterranean clover has thrown a somewhat different light on the earlier reports on plants showing estrogenic activity. It is now evident that the estrogenic properties of a plant may be due to the presence of either powerful or very weak estrogens, which need not necessarily be related to the known estrogens from animal sources, and that many plants previously reported estrogenic, but not examined further because of their relatively low potency, may well be worth further investigation. It is also clear that plants containing genistein and its derivatives are estrogenic, and possible that these compounds occur in plants other than those already investigated.

Hence, it is proposed to review all the information available on estrogens isolated from or shown, by tests, to be present in plants and to discuss these in the light of our present knowledge. Plant products closely related to known estrogens, either natural or synthetic, will also be surveyed in view of the possibility that such compounds may be converted to estrogens, either in the plant or subsequently, during the extraction process or after injection into the test animals. The estrogenic activity of some synthetic compounds related to genistein, prepared in the hope of eliciting information about its possible metabolism in the animal, will also be summarized.

No attempt will be made to survey work on the effect of estrogens on plants or on the substances which determine or influence the sex of plants. Although it is not unlikely that at least some plant estrogens have a sex-linked function in the plants in which they occur, we, as chemists, do not feel competent to discuss work in this field with authority.

II. Tests on Plant Extracts

In order to demonstrate the presence of estrogenic substances in plants, the first step has generally been to carry out tests with plant extracts injected subcutaneously into mice or rats and observe their effect by the Allen-Doisy technique. This is described in detail by Emmens (1950). A summary of the results obtained in investigations of this type is presented in Table I. An attempt has been made to compare the activities of the different plants examined by quoting the authors' results in rat or mouse units per kilogram. Solmssen (1945) pointed out that activities determined in different laboratories and at different times are not strictly comparable, and subsequent work has shown that such values may differ by a factor of 50, but no other means is available for giving a general indication of the order of effectiveness of the extracts examined in the earlier investigations. However, the limited accuracy should not be forgotten.

TABLE I

PLANTS REPORTED ESTROGENIC

Plant	Family	Part examined	Activity rat (R) or mouse (M) units/kg.	References
Aletris farinosa L.	Liliaceae	Roots	+	Costello and Lynn, 1950.
Allium sativum L. (Garlic)	Liliaceae	Bulb	4000 M	Glaser and Drobnik, 1939.
Alnus sp. (Alder)	Betulaceae	Catkins (male and female)	50 R	Walker and Janney, 1930.
Althaea rosea Cav. (Hollyhock)	Malvaceae	Leaves and stems	0.3–3 M	Loewe *et al.*, 1927.
Asclepias tuberosa L.	Asclepiadaceae	—	+; 1000 R	Costello and Lynn, 1950; Costello and Butler, 1950.
Avena sativa L. (Oats)	Gramineae	Seeds (meal)	+	Fellner, 1926.
		Seeds	6.6 R; 50 R	Walker and Janney, 1930; Butenandt and Jacobi, 1933.
		Sprouted seeds	50 R	Walker and Janney, 1930; Butenandt and Jacobi, 1933.
		—	5500 R; 16,000 R	Häussler, 1936.
Beta vulgaris L. (Beet)	Chenopodiaceae	Seeds	500 M	Dohrn *et al.*, 1926.
Brassica campestris L. (Rape)	Cruciferae	Seeds	+	Wadehn, 1928.
Butea spp.	Leguminosae	—	+	Schering-Kahlbaum A. G., 1935a, 1937a, b, c, d.
Butea superba Roxb.	Leguminosae	Tubers	900,000 M	Schering-Kahlbaum A. G., 1935b, 1938, 1939.
		Root	500,000 M	Vatna, 1939.
		Tubers	180,000 R	Schoeller *et al.*, 1940.
Chamaelirium luteum A. Gray (Helionas N.F.V.)	Liliaceae	Roots	+	Costello and Lynn, 1950.
Chrysactinia mexicana A. Gray	Compositae	Aerial parts	+	Lappas and Gustafson, 1950.
Cinnamomum zeylanicum Blume	Lauraceae	Bark	+	*Nøding et al., 1950.*

Coffea arabica L. (Coffee)	Rubiaceae	Seed (oil)	+	Slotta and Neisser, 1938; Paula, 1943; Chakravorty *et al.*, 1943.
Convallaria majalis L. (Lily of the Valley)	Liliaceae	Shoots	+	Schering, 1928.
Dactylis glomerata L. (Cocksfoot, orchard grass)	Gramineae	Leaf, stem, and flower	+	Legg *et al.*, 1950.
		—	−	Dohan *et al.*, 1951.
Dactylifera palmae L. (Date palm)	Palmaceae	Pollen	+	El Ridi and Wafa, 1947.
Elaeis guineensis Jacq.	Palmaceae	Kernel (residues)	20,000 M	Butenandt and Jacobi, 1933.
Eucalyptus sp.	Myrtaceae	Oil	+R 0 M	Zondek and Bergmann, 1938.
Foeniculum vulgare Mill. (Fennel)	Umbelliferae	Oil	+	Zondek and Bergmann, 1938.
Glycyrrhiza glabra L. (Licorice)	Leguminosae	Root	++	Costello and Lynn, 1950.
Grasses (pasture)	Gramineae	—	++ (spring)	Bartlett *et al.*, 1948.
		—	+ (summer)	
Helianthus annuus L. (Sunflower)	Compositae	Ovaries	+	Wehefritz and Gierhake, 1931.
Hordeum vulgare L. (Barley)	Gramineae	Embryo	+	Loewe, 1933.
Impatiens parviflora DC.	Balsaminaceae	Stems	0.3–1 M	Loewe *et al.*, 1927.
Lolium perenne L. (Ryegrass)	Gramineae	Leaves and stems	+	Legg *et al.*, 1950.
Malus sylvestris Mill. (=*Pyrus malus* L., apple)	Rosaceae	Fruit	+	Schering, 1928.
Nuphar luteum L. (Water Rose)	Nymphaeaceae	Ovaries	12–20 M	Loewe *et al.*, 1927.
Oryza sativa L. (Rice)	Gramineae	Seeds embryo	+	Fellner, 1926; Asikari, 1940.
Panax schin-seng Nees. (Ginseng)	Araliaceae	Root	+	Weber, 1938.
Petroselinum crispum Nym. (Parsley)	Umbelliferae	Root	+	Dohrn *et al.*, 1926; Schering, 1928.
Phoenix dactylifera L. (Date palm)	Palmaceae	Pollen	+	Hassan and Wafa, 1947.

TABLE I.—(*Continued*)

Plant	Family	Part examined	Activity rat (R) or mouse (M) units/kg.	References
Pimpinella anisum L.	Umbelliferae	Oil	+	Zondek and Bergmann, 1938.
Poa pratensis L. (Blue grass)	Gramineae	—	+ (weak)	Dohan *et al.*, 1951.
Prunus avium L. (Cherry)	Rosaceae	Fruit flesh	+	Dohrn *et al.*, 1926; Schering, 1928.
Prunus domestica L. (Plum)	Rosaceae	Fruit flesh	+	Dohrn *et al.*, 1926.
Rheum rhaponticum L. (Rhubarb)	Polygonaceae	Leaves	5 M	Walker and Janney, 1930; Schering, 1928.
Ricinus communis L. (Castor oil plant)	Euphorbiaceae	Seeds	+	Sahasrabudhe, 1945.
Saccharomyces sp. (yeast)	Ascomycetes (fungi)	—	250 M	Dohrn *et al.*, 1926.
			10 M	Dingemanse and Laqueur, 1927.
			+	Schering, 1928.
			30 M, 50 M	Glimm and Wadehn, 1928; *cf.* Loewe 1933.
Salix caprea L. (Willow)	Salicaceae	Flowers	48–200 M	Loewe *et al.*, 1927; *cf.* Wadehn, 1928; Walker and Janney 1930; Loewe, 1933.
		Stigma	8–14 M	Loewe *et al.*, 1927.
		Flowers	100 M, 33 M	Skarzynski, 1933a, b.
Salvia officinalis L. (Sage)	Labiatae	Leaves	6000 M	Kroszczynski and Bychowska, 1939.
Sambucus nigra L. (Elder)	Caprifoliaceae	Flowers	+	Much *et al.*, 1931.
Secale cereale L. var. Rosen (Rye grass)	Gramineae	—	+	Dohan *et al.*, 1951.
Solanum tuberosum L. (Potato)	Solanaceae	Tubers	+	Dohrn *et al.*, 1926; Wadehn, 1928; Schering, 1928.

TABLE I.—(*Continued*)

Plant	Family	Part examined	Activity rat = R, or mouse = M units/kg.	References
Tilia sp. (Linden)	Tiliaceae	Flowers	—	Much *et al.*, 1931.
Tillandsia usneoides L. (Spanish moss)	Bromeliaceae	—	+	Feurt and Fox, 1952.
Trifolium sp.	Leguminosae	—	+ +	Bartlett *et al.*, 1948.
Trifolium fragiferum L. (Strawberry clover)	Leguminosae	—	140 M	Robinson, 1949.
Trifolium pratense L. (Red clover)	Leguminosae	Leaves and stems	+	Legg *et al.*, 1950; Dohan *et al.*, 1951; Pope *et al.*, 1953.
Trifolium subterraneum L. var. Dwalganup (Subterranean clover)	Leguminosae	—	50 M (dry) (*per os*)	Curnow *et al.*, 1948.
		—	1000 M (dry)	Robinson, 1949.
		Leaves	+ +	
		Petioles	+	
		Roots	+	
Trifolium subterraneum L. var. Mt. Barker (Subterranean clover)	Leguminosae	—	+	Robinson, 1949.
Triticum aestivum L. (Wheat)	Gramineae	Seeds (flour)	+	Fellner, 1920
		Seeds	+	Wadehn, 1928.
		—	−	Dohan *et al.*, 1951.
		Germ oil	+	Levin *et al.*, 1951.
Tulipa sp. (Tulip)	Liliaceae	Bulbs	500 M	Coussens and Sierens, 1949.
Urtica sp. (Nettle)	Urticaceae	Flowers	+	Much *et al.*, 1931.
Viola odorata L.	Violaceae	—	+	Giacomello, 1938.

In addition to the observations made by the Allen-Doisy technique, the results obtained with subterranean clover (Curnow *et al.*, 1948; Robinson, 1949) and grasses (Bartlett *et al.*, 1948) by measuring the increase in uterine weight caused by injecting extracts into mice are shown in Table I in semiquantitative form. These should not be correlated with figures obtained by the Allen-Doisy method.

In all, over 40 species have been shown to have some estrogenic potency, but although the activity has in a few cases been confirmed by several groups of investigators, very few plants have yielded crystalline estrogens. Estrogen has also been reported in bacteria (Pedersen-Bjergaard, 1933), and in a commercial animal ration (Zarrow *et al.*, 1953).

The high activities reported for extracts of *Asclepias tuberosa*, *Butea superba*, garlic, sage, and tulips are particularly striking, but in spite of this only the *Butea* extracts have been purified sufficiently to yield a crystalline estrogen, although further work is no doubt proceeding on *Asclepias tuberosa*. Further investigation of this highly active group should be most productive, as even in the case of the *Butea* estrogen no structural studies have yet been reported.

TABLE II

NON-ESTROGENIC PLANTS

Plant	Family	Reference
Cimicifuga racemosa Nutt.	Ranunculaceae	Costello and Lynn, 1950.
Cocos nucifera L. (Coconut)	Palmaceae	Much *et al.*, 1931.
Cryptostemma calendulaceum R. Br. (Capeweed)	Compositae	Bennetts and Underwood, 1949.
Cyparissias sp.	Euphorbiaceae	Much *et al.*, 1931.
Ehrharta calycina Sm.	Gramineae	Robinson, 1949.
Erodium botrys Bertol. (Flatweed)	Geraniaceae	Bennetts and Underwood, 1949.
Gentiana lutea L.	Gentianaceae	Costello and Lynn, 1950.
Juglans sp. (Walnut)	Juglandaceae	Much *et al.*, 1931.
Lolium rigidum Gaud. (Wimmera ryegrass)	Gramineae	Bennetts and Underwood, 1949.
Matricaria chamomilla L.	Compositae	Costello and Lynn, 1950.
Medicago denticulata Willd.	Leguminosae	Robinson, 1949.
Medicago sativa L. (Alfalfa, lucerne)	Leguminosae	Legg *et al.*, 1950, *cf.* Dohan *et al.*, 1951.
Phleum pratense L. (Timothy grass)	Gramineae	Legg *et al.*, 1950.
Senecio aureus L.	Compositae	Costello and Lynn, 1950.
Taraxacum sp.	Compositae	Costello and Lynn, 1950.
Trifolium repens L. (White clover)	Leguminosae	Robinson, 1949; Legg *et al.*, 1950.

The plants listed in Table II did not show estrogenic activity when extracts were tested. Extracts of the white bean (*Phaseolus vulgaris* L., Leguminosae) were even reported to inhibit estrus (Belak and Szathmary, 1937; Zsclonka and Illenyi, 1937).

Systematic examination of a much wider group of plants for estrogenic constituents should also be fruitful. It is very likely that estrogens of similar structure will be found, like most other groups of plant products, such as alkaloids, terpenes, and lignans, in a limited number of families, with closely related species containing identical or very closely related compounds.

Screening tests could easily be run with a few grams of material (*cf.* Bartlett *et al.*, 1948) and the more promising plants investigated in bulk. Chromatographic and spectrographic methods for the separation and identification of the constituents of plant extracts put the present-day investigator in a much more favorable position than his predecessors, even of ten years ago, properly to characterize and identify minute quantities of material. Furthermore the isolation of genistein has demonstrated that plant estrogens are not necessarily highly potent compounds like estrone, and if the estrogen is relatively weak a given potency in the plant represents a much larger weight of active material, and the problem of isolation is considerably simplified.

III. Isolation of Estrogens

Estrogenic constituents have now been isolated from six plants and it is clear that although these constituents are in some cases identical with normal animal estrogens, this identity is by no means universal. Estrone was isolated by Butenandt and Jacobi (1933) from palm kernel residues and estriol from willow catkins (Skarzynski, 1933a, b). A formula ($C_{19}H_{22}O_8$) has been suggested for the *Butea superba* estrogen (Schering-Kahlbaum A. G., 1938; Butenandt, 1940), but little evidence is yet available to support this, and there is no indication whether it belongs to any known class of plant products or not.

The estrogen of *Trifolium subterraneum* has recently been completely characterized (Bradbury and White, 1951) and its identification as genistein (III) indicates that several other plants, which are already known to contain genistein, and its weakly estrogenic 7-methyl ether, prunetin (Table III), must also be estrogenic. It is not surprising, therefore, that Cheng *et al.* (1953) have obtained estrogenic effects with genistein and its 7-glucoside (genistin) from soya beans and that Pope *et al.* (1953) were able to demonstrate the estrogenic effect of genistein 4′-methyl ether, isolated from red clover.

1. Palm Kernels

The first isolation and characterization of an estrogen from a plant source was accomplished by Butenandt and Jacobi (1933). They extracted the residual cake left on pressing "palm kernels," and although they did not specify the type of palm from which the kernels were obtained it is almost certain to have been *Elaeis guineensis*, as this is the only palm, apart from the coconut, used commercially for oil production. From 50 kg. of the press-cake they obtained 2.4 kg. of oil, soluble in methanol and with an estrogenic activity (1 million mouse units) equivalent to 100 mg. of estrone. This oil was saponified, the nonsaponifiable fraction distilled and hydrolyzed with acid. The phenolic fraction was then separated and partitioned between aqueous methanol and petroleum ether. From the aqueous solution 85% of the original activity was recovered, and on distillation the product crystallized on rubbing with ethyl acetate; after crystallization from aqueous alcohol 18 mg. of estrone were obtained. This was thoroughly characterized by melting point, optical rotation, ultraviolet absorption maximum at 283 to 285 mμ, and by the preparation of the benzoate and semicarbazone, which were compared with authentic samples. In addition, the biologic activity was found to be equal to that of estrone isolated from urine.

This most meticulous piece of work should be considered a model for the characterization of all products isolated from plants, with the addition in future work of a determination of the infrared absorption spectrum and measurement of the intensity of the ultra-violet absorption.

2. Willow Catkins

Willow catkins were first reported to contain estrogen by Loewe (1926), and Loewe *et al.* (1927) reported that the fresh flowers contained 200 mouse units per kilogram. Closely following Butenandt and Jacobi's isolation of estrone from palm kernels, Skarzynski (1933a, b) reported the isolation of a crystalline estrogen from willow flowers. Using flowers collected in 1932, he obtained 7.5 mg. of crystals from 65 kg. of flowers and found that these resembled estriol in microscopic appearance, solubilities, ultraviolet absorption, and melting point. Their m.p. was depressed only 1° C. by estriol of melting point 277° C. isolated from urine, and the melting points of the respective acetates were identical.

The biologic activity of Skarzynski's product was, however, only one-quarter that of the estriol he prepared for comparison, from urine, but this difference is probably not significant in view of the limited accuracy of the Allen-Doisy technique at that time.

Reinvestigation of the estrogens from this plant could clear up this

point and perhaps amplify Skarzynski's report (1933a) that a more active yellow substance separated from the mother liquors of his estriol. However, the poor yield obtained by Skarzynski (1933b) from flowers collected in the 1933 season (5.5 mg. from 165 kg.) does not hold out much prospect of success unless very large quantities of flowers are available, although it may be possible to obtain better yields by employing chromatographic methods for the isolation.

3. *Butea superba*

Following claims in the patent literature (Schering-Kahlbaum A. G., 1935a, b; 1937a, b, c, d; 1938, 1939) covering the extraction and concentration of a highly active estrogen from *Butea* spp. assaying at 900,000 mouse units per kilogram of tubers subcutaneously, or 450,000 *per os*, Vatna (1939) reported that the storage root of *Butea superba* Roxb. contained estrogenic material extractable by alcohol or water. The potency was equivalent to 500,000 mouse units per kilogram on extraction with alcohol, but the aqueous extract was less potent and more toxic.

This observation prompted the publication by Schoeller *et al.* (1940) describing their results with extracts of *Butea superba*, which had originally been brought to their notice because of its use as an aphrodisiac. They observed estrogenic activities as high as 180,000 rat units per kilogram and isolated the estrogenic substance in crystalline form. They did not describe the methods used in the isolation but noted that alkaline hydrolysis, as employed in the isolation of estrone, cannot be used on *Butea* extracts, as the estrogen is easily decomposed by alkali. Their product melted at ca. 260° C. and only twice the subcutaneous dose was required for activity *per os*, in contrast to estrone and estradiol which they found required 73 and 450 times the subcutaneous dose, respectively. Subcutaneously, the activity of the *Butea* estrogen was about midway between that of estrone and of estradiol, and its effects were completely parallel to those of the natural hormones.

Chemical investigation of the *Butea* estrogen by Butenandt (1940) showed that it was weakly acidic and reduced Fehling's solution; he claimed that it had the molecular formula $C_{19}H_{20}O_6$, formed a monomethyl ether, m.p. 288–290° C., and in the presence of hydrochloric acid gave an anhydro-derivative said to be $C_{19}H_{20}O_5$, m.p. ca. 350° C. In view of the formula attributed to the anhydro-derivative it seems more likely that the formula of the estrogen is $C_{19}H_{22}O_6$ as claimed by Schering-Kahlbaum A. G. (1938). This patent also quotes the melting point as 270° C.

The estrogen was accompanied by a physiologically inactive weakly acidic substance, m.p. 320° C., which was considered likely to be chemi-

cally related to the estrogen, but no further reports have been published on the constitutions of either of these compounds.

The isolation of this estrogen marks an important advance in our knowledge of plant estrogens, as it was the first demonstration of the presence in a plant of a highly potent substance, not identical with the normal steroid estrogens of the animal kingdom. It is most unfortunate that investigation of this extremely interesting compound appears to have been discontinued, and it is sincerely to be hoped that further study of its constitution will be undertaken in the near future.

4. Subterranean Clover

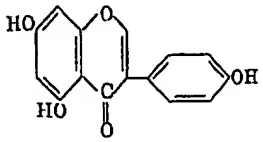
Field observations and laboratory studies (Bennetts, 1944) led to the realization of the serious implications of a breeding problem in sheep grazing on subterranean clover pastures. It was shown that these breeding difficulties were associated with the early Dwalganup strain of subterranean clover (*Trifolium subterraneum* L.) and that its manifestations included infertility, dystocia, and prolapse of the uterus, some months after parturition or even in unmated ewes.

Bennetts *et al.* (1946) provided strong presumptive evidence of the presence in this pasture plant of an estrogen or a proestrogen (*cf.* Emmens, 1941). This was confirmed by Curnow *et al.* (1948), who showed that the ether extract of the clover gave, *per os*, qualitatively similar effects to estradiol given subcutaneously. The estrogen was found to be nonvolatile and ether-soluble and was extracted from ether by sodium hydroxide solution. The ether extract from 5 g. of dried clover *per os* was somewhat less potent than 0.04 microgram of estradiol given subcutaneously.

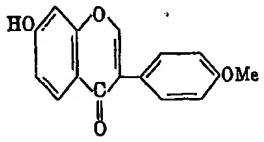
Further studies by Robinson (1949) showed that estrogens were present in the Mt. Barker as well as in the Dwalganup strain of subterranean clover and also in one sample of strawberry clover (*Trifolium fragiferum* L.) but the other pasture plants tested, *Medicago denticulata* Willd., *Ehrharta calycina* Sm., and white clover (*T. repens* L.) were not estrogenic. Extraction, concentration, and assay of the subterranean clover estrogen were studied, and it was shown that more activity was contained in the leaves than in the roots or petioles. Later 20 different strains were reported estrogenic (Bennetts and Underwood, 1949), and East (1950) showed that 30 g. dry subterranean clover produced an increase in nipple length in male guinea pigs similar to that caused by 2.5 micrograms of stilbestrol, while Legg *et al.* (1950) showed that the estrogen could be conveniently concentrated with the chloroplast material from the plant by heating or centrifuging the press-juice. This concentration enabled the convenient preparation of extracts by means of organic solvents. Curnow (1950) and Beck and Braden (1951) studied these

extracts by methods involving saponification with alcoholic alkali, but although they obtained potent fractions they were unable to isolate and characterize any pure compounds.

In view of the possibility that alkaline hydrolysis might lead to decomposition, as in the case of the *Butea* estrogen, and also the observation that such a procedure led to the presence of substantial quantities of fatty acids in the sodium hydroxide extracts, Bradbury and White (1951) studied an alcoholic extract of the "chloroplast fraction" by chromatography on alumina. Two crystalline products were isolated and identified as the isoflavones, formononetin (IV) and genistein (III). Formononetin showed no estrogenic activity, but genistein was active at 1 mg. in



III



IV

mice, and Biggers (1951) reported that it behaved as a proestrogen (*cf.* Biggers and Curnow, 1954).

Although only 20 mg. of genistein were isolated per kg. of fresh clover, considerable quantities would have remained in the press-residue and in the aqueous solution after removal of the "chloroplast fraction"; thus it was considered that genistein "must be the principal estrogen in the clover." This conclusion has been confirmed by subsequent work (Curnow, 1954). Further investigation of saponified extracts has resulted in the isolation from them of genistein, together with its 4′-methyl ether (biochanin-A) and a fraction of greater estrogenic activity which has not yet been obtained pure. They also contain 2,4-dihydroxyphenyl *p*-methoxybenzyl ketone (ononetin), the saponification product of formononetin (Beck *et al.*, 1954).

5. Other Plants

Genistein and its 7-glucoside (genistin) isolated from soya beans have also been shown to be estrogenic (Cheng *et al.*, 1953) while the 4′-methyl ether of genistein (biochanin-A) was shown to be estrogenic by Pope *et al.* (1953), who isolated it from red clover.

The pollen grains of the date palm (*Phoenix dactylifera* L.) were extracted by Hassan and Wafa (1947) using Marrian's method, and their water-soluble product showed color reactions and ultraviolet absorption resembling estrone. Similarly, El Ridi and Wafa (1947), using the procedure of Butenandt and Jacobi (1933), obtained from *Dactylifera*

palmae L., an oil insoluble in water and showing 40% of the estrogenic activity of estradiol propionate. This oil gave color reactions similar to estrone and had its maximum ultraviolet absorption at a wavelength similar to that of the estrogenic hormones (282 mμ). It is unfortunate, in view of the high activity of the extract, that they were not able to obtain a crystalline product or characterize the active substance by the preparation of a crystalline derivative.

Similarly estrogenic concentrates have been obtained by Costello and Lynn (1950) from licorice root and by Costello and Butler (1950) from *Asclepias tuberosa* L., but they have not described any pure compound showing estrogenic activity.

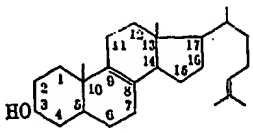
The correlation of the wavelength of the absorption maxima in the ultraviolet spectra of the licorice extracts and the date palm pollen extracts with those of estriol and other steroid estrogens may well be fortuitous, as an absorption band at 280 to 282 mμ is by no means specific for estrogenic phenols. Such a band is in fact a characteristic feature of the spectra of many benzenoid compounds in which the characteristic benzene band at 256 mμ is displaced to a longer wavelength by the presence of an "auxochromic" substituent (*cf.* Braude, 1945). It is even present in such simple compounds as aniline and anisole (Braude, 1945) and is also seen in isoflavone reduction products such as 4',5,7-trimethoxyisoflavanone ($\epsilon_{max.}$ 20580 at 283.5 mμ; Bradbury and White, 1953) and 4',7-dimethoxyisoflavan ($\epsilon_{max.}$ 5500 at 282 mμ; Wessely and Prillinger, 1939). Its diagnostic value is therefore very small, particularly in the absence of any intensity measurements.

The estrogens reported in honey by Dingemanse (1938) and Serono and Montezemolo (1941) may also be of plant origin.

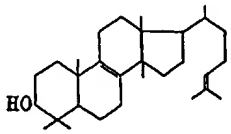
IV. Related Plant Products

1. Steroids

The presence of sterols in a large number of plants has been quite well established, although in a number of cases they have not been completely purified or adequately characterized. The better characterized compounds are reviewed by Fieser and Fieser (1949), and it is noteworthy that sterols have been isolated from several of the plants listed as estrogenic in Table I. Yeast, particularly, contains a wide variety of sterols including ergosterol, zymosterol (V) and others of normal sterol type, and in addition lanosterol (kryptosterol) (VI), which, although it resembles the triterpenes in having 30 C atoms and a *gem*-dimethyl group on C_4 in ring A, has now been classed as a sterol (Voser *et al.*, 1952). It would not be at all surprising if these sterols were accompanied by small amounts of



V



VI

steroid estrogens, either as normal plant constituents, or perhaps formed by some biologic oxidation process from the sterols. Alternatively it may be possible for test animals to transform certain sterols in part into estrogens, or the estrogens may arise by oxidation of the sterols, during the course of the isolation procedure.

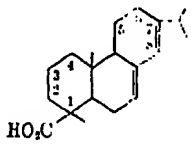
It may be significant that the preferred sources of plant sterols are often the seeds (*e.g.*, wheat germ oil, rape seed oil, alfalfa seed, corn oil, rye germ oil, soya bean oil), while Walker and Janney (1930) have pointed out that sprouted oats is a rich source of estrogen and make the generalization that rapidly growing plant material is richest in estrogen. This, however, is not supported by Butenandt and Jacobi (1933), who were unable to detect any increase in the estrogenic potency of oats after germination, with the result that the attractive hypothesis that the sterols in the seeds are converted into estrogens on germination is at present without any foundation.

In addition to the sterols themselves, other compounds with steroid nuclei found in plants include the cardiac glycosides, the steroid saponins, and some alkaloids. Cardiac glycosides have been reported in plants belonging to the families Apocynaceae, Asclepiadaceae, Celastraceae, Liliaceae, Ranunculaceae, and Scrophulariaceae (*cf.* Fieser and Fieser, 1949; Hauenstein *et al.*, 1953, and earlier papers), whereas steroid saponins are found in the Liliaceae, Amaryllidaceae, Dioscoreaceae, Bromeliaceae, and Scrophulariaceae (Marker *et al.*, 1947). Of these families four, namely, Amaryllidaceae, Asclepiadaceae, Bromeliaceae, and Liliaceae, are represented among those reported to contain estrogens (Table I), and it is possible that there may be some connection between the presence of estrogen and the occurrence of these steroids.

Among the plants known to contain steroid alkaloids, only the potato has been reported estrogenic and this activity could not be confirmed (Walker and Janney, 1930). Thus there seems to be no indication of any connection between steroid alkaloid content and estrogenic activity.

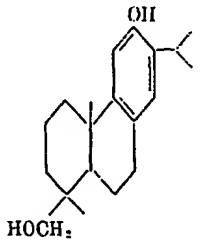
2. Other Hydrophenanthrene Compounds

Fieser and Campbell (1939) reported that 6-hydroxydehydroabietinol (VIII), prepared from abietic acid (VII), showed slightly greater estro-

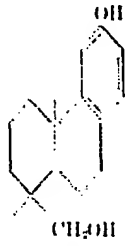


VII

genic activity than estrone in a 5-microgram dose in rats but was less active at 20-micrograms, presumably because of toxic effects. The related podocarpinol (IX), obtained independently by Brandt and Ross



VIII



IX

(1948) and by Zeiss *et al.* (1948), which differs from 6-hydroxydehydroabietinol only in the absence of the 7-isopropyl substituent and in the configuration of the groups attached to C_1, was also found to be estrogenic (Brandt and Ross, 1948). Baizer *et al.* (1950) showed that 7-isopropylpodocarpinol was also estrogenic in doses similar to those required with its 1-epimer (VIII). Thus it is clear that in this group of compounds the estrogenic potency is not substantially affected by either the configuration at C_1 or by the presence or absence of an alkyl substituent on C_7.

The resin acids from which these estrogens are derived are characteristic constituents of pine resins, abietic acid (VII) and its isomers *d*-pimaric acid and *l*-pimaric acid being obtained from a variety of *Pinus* spp., while podocarpic acid occurs in a Javanese *Podocarpus* and New Zealand *Podocarpus* and *Dacrydium* spp. (Sherwood and Short, 1938). In view of the indication above that the presence of estrogenic activity in compounds related to these diterpenoids does not require very rigid stereochemical or constitutional relationships it would appear to be worth examining a wider range of related compounds and perhaps also worth seeking estrogenic fractions among the resin constituents themselves.

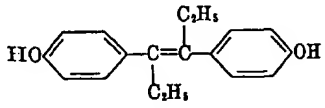
Crude samples of cafestol ($C_{20}H_{28}O_3$; *cf.* Haworth *et al.*, 1954), formerly known as cafesterol, and other less pure fractions from the un-

saponifiable fraction of coffee oil have been reported estrogenic (Table I), but no such activity was found by Wettstein *et al.* (1941) or by Hauptmann and França (1943) with purer samples of cafestol. In fact, Hauptmann *et al.* (1943a, b) were unable to detect estrogenic activity in any coffee-oil fraction. Hence it seems likely that the estrogen is an artefact, formed under some conditions of isolation.

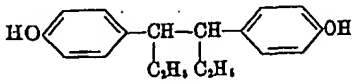
A triterpenoid constituent of violet wax has also been reported estrogenic (Giacomello, 1938).

3. Stilbenes

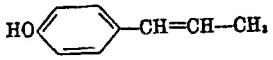
The very powerful estrogenic effects of the synthetic compounds stilbestrol (X) and hexestrol (XI) have led to speculation on the possibility of the occurrence of related compounds in plants. Deulofeu (1941b)



X



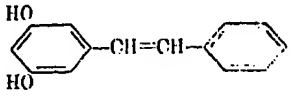
XI



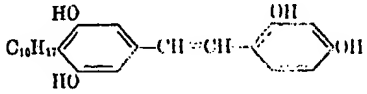
XII

suggested that the estrogenic activity of some plant extracts, at least, might be due to the presence of transformation products of anol (XII) or its methyl ether, anethole. Zondek and Bergmann (1938) had already shown that anise and fennel oils, which are rich in anethole, had some estrogenic activity, but whether this is due to anethole itself or to traces of highly active transformation products has never been satisfactorily established.

Other stilbene derivatives have, however, been isolated from plants, notably the compounds pinosylvin (XIII) and its methyl ethers, which are common constituents of the heartwoods of trees belonging to the genus *Pinus* (Lindstedt, 1951), and chlorophorin (XIV), which occurs in the African tree, *Chlorophora excelsa* Benth. et Hook. f. (Moraceae) (King and Grundon, 1949, 1950; Nunn and Rapson, 1949).



XIII



XIV

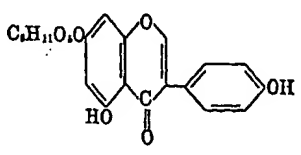
It may well be that the pine constituents have no estrogenic activity since they do not contain a 4- or 4'-hydroxyl group, although even stilbene itself has been reported estrogenic in 25 mg. doses in rats and the presence of one or two *para*-hydroxyl groups only reduces the dose required to 10 mg. (Solmssen, 1945). However, estrogenic activity of at least this low order is to be expected of chlorophorin.

4. Isoflavones

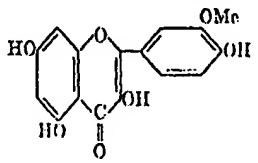
The list of naturally occurring isoflavones (Warburton, 1954) clearly shows the limited occurrence of isoflavones in contrast to the closely related flavones. This apparent scarcity, however, may be due in part to failure of the methods employed in investigating plants to extract and purify the isoflavones. Three of the naturally occurring compounds, genistein (III), its 7-methyl ether prunetin, and its 4'-methyl ether biochanin-A, have been shown to be estrogenic (Bradbury and White, 1951, 1953; Pope *et al.*, 1953) and plants containing these compounds and their glycosides are listed in Table III on page 225.

It is probable, too, that many of the plants in Table I, in addition to subterranean and red clovers, owe their estrogenic potency to the presence of isoflavones. This is particularly likely where the plants belong to families from which estrogenic isoflavones have already been isolated (Table III), although it would be surprising if the estrogen from *Butea* spp. were an isoflavone in view of its very great potency (Butenandt, 1940).

The observation of Kuhn *et al.* (1944) that genistin (genistein 7-glucoside) (XV) and its derivatives immobilize the gametes of *Chlamydomonas*,



XV



XVI

TABLE III

PLANTS CONTAINING GENISTEIN, PRUNETIN, OR BROCHANIN-A

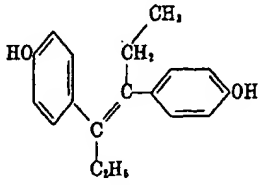
Plant	Family	Compound	Reference
Cicer arietinum L.* (*Chana* grain)	Leguminosae	Biochanin-A (Genistein 4'-methyl ether)	Siddiqui, 1945; Bose and Siddiqui, 1945.
Ferreirea spectabilis Allem.	Leguminosae	Biochanin-A	King *et al.*, 1952.
Genista tinctoria L.	Leguminosae	Genistin (Genistein 7-glucoside)	Perkin and Newbury, 1899; Zemplen and Farkas, 1943.
Soja hispida L.	Leguminosae	Genistin	Walz, 1931; Walter, 1941; Cheng *et al.*, 1953; Ahluwalia *et al.*, 1953.
Sophora japonica L.	Leguminosae	Sophoricoside (Genistein 4'-glucoside)	Charaux and Rabaté, 1935, 1938; Zemplen, *et al.*, 1943.
		Sophorabioside (Genistein 4'-*l*-rhamnosido-*d*-glucoside)	Zemplen and Bognar, 1942.
Prunus sp.	Rosaceae	Prunetin glucoside	Finnemore, 1910.
Prunus puddum Roxb.	Rosaceae	Prunetin	Chakravarti and Bhar, 1945; King and Jurd, 1952.
Pterocarpus angolensis DC	Leguminosae	Prunetin	King and Jurd, 1952.

* It seems likely that this was the species investigated, although *Panicum colonum* L. (Gramineae) is also known by a similar colloquial name.

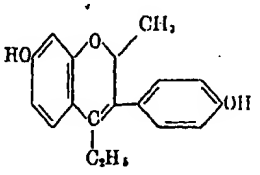
although they do not possess the sex-determining power of isorhamnetin (XVI), is of great interest in view of the estrogenic activity of genistein in animals. Whether there is any connection between these two effects remains to be determined. Further investigation might help in establishing the function of genistein in the plant.

V. Synthetic Compounds Related to Genistein

In view of the report (Biggers, 1951) that genistein was a proestrogen, the activity of which would probably be due to its metabolism in the animal body to the true estrogen, a number of related compounds were prepared in the hope of obtaining some indication of the nature of the true estrogen. It seemed possible that more active compounds might result from transformations which produced molecular structures more closely resembling stilbestrol (XVII). In order to obtain such structures



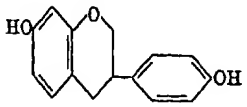
XVII



XVIII

from genistein (III) a number of modifications of its structure are required, and the compound (XVIII) is presumably the closest approach to stilbestrol which can be achieved without removing the oxygen of the heterocyclic ring. A variety of intermediate structures between genistein (III) and XVIII have been examined (Tables IV and V). In addition to these derivatives, other possible routes to compounds which behave as true estrogens include the formation of isoflavylium salts and compounds derived from these and, rather less likely, the substituted phenyl benzyl ketones formed by treatment of isoflavones with alkali.

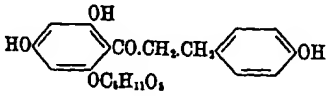
The possible importance of the oxonium (isoflavylium) salts as intermediates is emphasized by the consideration of the important roles of ammonium and sulfonium salts in enzyme-catalyzed reactions (Woolley, 1953). Taken in conjunction with the isolation of the isoflavone reduction product, equol (XIX) from horses' urine (Marrian and Haslewood, 1932),



XIX

this would make it appear very likely that isoflavones suffer reduction in biologic systems. However, equol and the simple reduction products which we have tested are not estrogenic, so that there is as yet no direct evidence that estrogenic activity is produced by reduction of genistein or its derivatives.

The possibility of producing true estrogens by treatment of isoflavones with alkali seems less likely, as 2,4,6-trihydroxyphenyl *p*-hydroxybenzyl ketone is not estrogenic in mice at a total dose of 1.1 mg. (Bradbury, 1953b), although the related compound phlorhizin (XX) has been shown to be weakly estrogenic (Dodds and Lawson, 1938).



XX

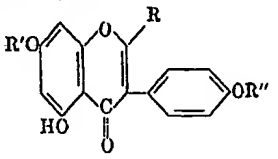
1. *Isoflavones*

The first compounds prepared were isoflavones either lacking the 5-hydroxyl or with a 2-alkyl substituent or both, as well as some of their methyl ethers (Table IV).

TABLE IV

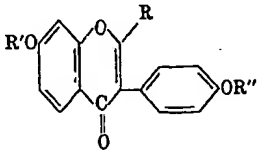
Isoflavone	Estrogenic activity (total dose per mouse)	
	Active at mg.	Inactive at mg.
4',5,7-Trihydroxy- (Genistein) (III)	1.0	—
2-Methyl-4',5,7-trihydroxy- (XXI)	—	8.3
2-Ethyl-4',5,7-trihydroxy- (XXII)	—	3.3
4',5-Dihydroxy-7-methoxy- (Prunetin) (XXIII)	2.1	—
5-Hydroxy-4',7-dimethoxy- (XXIV)	—	5.0
4',7-Dihydroxy- (Daidzein) (XXV)	—	5.4
2-Methyl-4',7-dihydroxy- (XXVI)	—	6.6
2-Ethyl-4',7-dihydroxy- (XXVII)	—	12.6
4'-Methoxy-7-hydroxy- (Formononetin) (XXVIII)	—	2
2',5,7-Trihydroxyisoflavone[a]	4	—

[a] Baker *et al.*, 1953.



III and XXI–XXIV

III	R = R' = R'' = H
XXI	R = Me, R' = R'' = H
XXII	R = Et, R' = R'' = H
XXIII	R = R'' = H, R' = Me
XXIV	R = H, R' = R'' = Me



XXV–XXVIII

XXV	R = R' = R'' = H
XXVI	R = Me, R' = R'' = H
XXVII	R = Et, R' = R'' = H
XXVIII	R = R' = H, R'' = Me

Estimates of activity are only preliminary, being determined by uterine weight increase on groups of five to six mice. Injections were given morning and evening on each of three days and uteri fixed and weighed next morning.

However, it is clear that in this group of compounds there is no resemblance in estrogenic activity to the stilbestrol model, for daidzein and its 2-alkyl derivatives are quite inactive at the largest doses which could be conveniently given in oil solution (or in some cases suspension),

while even the introduction of a 2-alkyl group into the genistein molecule removes its activity. Methylation of the 7-hydroxyl reduces the potency but this effect is to be anticipated with almost any phenolic estrogen.

In reporting these results Bradbury and White (1953) noted that the introduction of a methyl group in the 2-position in genistein resulted in a reduction of the intensity of ultraviolet absorption and caused the maximum to move to a slightly shorter wavelength, and suggested that this indicated a deformation of the planar genistein molecule. From this it was inferred that the presence of estrogenic activity in genistein was correlated with the coplanarity of the molecule.

The presence of the 5-hydroxyl group appears to be quite important, since it is present in all four estrogenic isoflavones prepared to date, namely, genistein (III), prunetin (XXIII), biochanin-A, and 2',5,7-trihydroxyisoflavone. Probably its importance is related to the fact that it modifies the properties of the carbonyl group. This type of effect (*cf.* Davies, 1953), previously ascribed to hydrogen bonding and chelate-ring formation has been shown by Hergert and Kurth (1953) to be due partly to mesomerism which decreases the double-bond character of the carbonyl C=O linkage by partial formation of $\overset{\diagdown}{\underset{\diagup\!\diagup}{C}}\!-\!\bar{O}$. The presence of the latter structure increases the intensity of the carbonyl band in the infrared spectrum and may also be responsible for the relatively high intensity ($\epsilon_{max.}$ 42,700) of the genistein absorption band at 262 mμ.

2. *Isoflavanones*

Reduction of the 2,3 double bond in the isoflavones was accomplished by the use of Adams' platinum catalyst in acetic acid solution and in this way derivatives of 4',7-dihydroxy-, 2-methyl-4',7-dihydroxy-, and 4',5,-7-trihydroxy-isoflavanones were obtained (Bradbury and White, 1953) but they did not produce estrus in mice in doses of 1–2.5 mg.

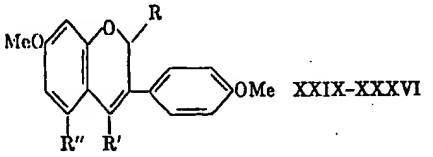
3. *Isoflavens*

Some isoflavens (Table V) were prepared by further hydrogenation of the isoflavanones with platinum oxide in acetic acid, intermediate isoflavanols being isolated in two cases. Others were obtained by the action of Grignard reagents on the isoflavanones, and the position of the double bond in the heterocyclic nucleus was established as 3,4 from the ultraviolet absorption spectra. Although the 4',7-dimethoxy-compound (XXIX) was not estrogenic, the homolog with a 2-methyl substituent

TABLE V

Isoflaven	Estrogenic activity[c] (total dose per mouse)	
	Active at mg.	Inactive at mg.
4',7-Dimethoxy-[b] (XXIX)	—	4.1
2-Methyl-4',7-dimethoxy-[b] (XXX)	0.07	—
4-Methyl-4',7-dimethoxy-[b] (XXXI)	0.35	—
4-Ethyl-4',7-dimethoxy-[b] (XXXII)	0.07	—
4-Phenyl-4',7-dimethoxy-[a] (XXXIII)	0.012	—
4-*p*-Methoxyphenyl-4',7-dimethoxy-[a] (XXXIV)	0.026	0.005
4-Phenyl-4',5,7-trimethoxy-[a] (XXXV)	0.68	—
4-*p*-Methoxyphenyl-4',5,7-trimethoxy-[a] (XXXVI)	0.44	—

[a] Bradbury, 1953a.
[b] Bradbury and White, 1953.
[c] Estimated as for the compounds in Table II.



XXIX–XXXVI

XXIX R = R' = R'' = H
XXX R = Me, R' = R'' = H
XXXI R = R'' = H, R' = Me
XXXII R = R'' = H, R' = Et
XXXIII R = R'' = H, R' = Ph
XXXIV R = R'' = H, R' = *p*-$MeOC_6H_4$-
XXXV R = H, R' = Ph, R'' = MeO-
XXXVI R = H, R' = *p*-$MeOC_6H_4$-, R'' = MeO-

(XXX) was a powerful estrogen, much more potent than genistein, and showing clearly the close approach to the stilbestrol model. Similar high potency was obtained with the 4-ethylisoflaven (XXXII), which as expected from analogy with the stilbestrol series was more active than the lower homolog (XXXI). Resemblance to the triphenylethylene estrogens was obtained by the introduction of aryl substituents at C_4 and even more potent products obtained.

Thus it is clear that the isoflavens, unlike the isoflavones, are closely comparable to the stilbene estrogens, since the effects of substituent groups on their activity are almost identical with the effects of similar groups in the stilbene series (*cf.* Solmssen, 1945). The contrast with the isoflavones is emphasized by the reduction in activity on introduction

of a 5-methoxyl group in the isoflavens, whereas a 5-hydroxyl is apparently essential for any activity in the isoflavone series.

VI. General Summary

It is quite obvious that our knowledge of plant estrogens is as yet limited and fragmentary. Clearly a great deal more systematic investigation is required on plant extracts to select the more promising sources of estrogens, followed by the isolation and characterization of the estrogens themselves, before we are able to generalize about their occurrence in the various plant families or about their nature. Concurrently, investigation of the functions in the plant of the compounds, which can be isolated, is urgently required, as our knowledge of this field of plant physiology is as yet seriously deficient. If knowledge of this field could be extended sufficiently, it would be a very valuable aid to prediction of the nature and the type of plant in which estrogens might be found.

The present position can be summarized by saying that the known plant estrogens are estrone, estriol, and genistein and its derivatives, while the *Butea* estrogen, $C_{19}H_{22}O_6$, is as yet unidentified. The active compounds in some 40 other plants, reported estrogenic, have not yet been isolated or characterized and it is highly probable that many other plants contain estrogenic compounds. They should provide a fertile field for future investigation.

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REPRODUCTIVE AND GENERAL METABOLIC EFFECTS OF PHYTOESTROGENS IN MAMMALS

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INTRODUCTION

Historically, phytoestrogens were first investigated when it was noted that ewes that grazed Australian clover pastures for prolonged periods of time became sterile. It was found that the active agents in the clover that precipitated sterility were estrogenic (1). Later a similar phenomenon was observed to occur in the California quail during dry years, when phytoestrogen concentrations in available forage were increased (2).

Phytoestrogens are defined as plant substances that are structurally and functionally similar to the gonadal steroid 17β-estradiol (E_2) or that produce estrogenic effects (3). There are three main groups of nonsteroidal dietary estrogens. Phytoestrogens include the isoflavones (i.e., genistein, genistin, daidzein, biochanin A, formononetin, and pratensein) and the coumestans (i.e., coumestrol and 4'-o-methylcoumestrol). Mycoestrogens of the resorcylic acid lactone group (i.e., zearalenone and zearalenol) are also commonly found (4). The structural similarity between these substances, endogenous mammalian estrogens (E_2 and estrone), and potent synthetic estrogens (diethylstilbestrol) have been studied (Figure 1). Isoflavones, the monocarboxylic derivatives of the 15-C flavones, and coumestans contain central structures of 15 carbons. Both of these groups are derivatives of 3-phenylchroman (Figure 1) and thus may be considered a single family of compounds (5). The fungal resorcylic acid lactones and endogenous estrogens possess central structures of 17 carbons.

The similarity among these compounds has led investigators to study the possibility that phytoestrogens might act on physiological processes and behavioral patterns to alter reproductive performance (3). If reproductive effects occur, then these compounds might have a role in the evolutionary success of herbivores, perhaps making the difference between survival and extinction for some species. It is possible that phytoestrogens, through mimicry of endogenous animal estrogens, function as defensive substances by which plants diminish the fertility of herbivores which feed on the plants (6). In effect, the phytoestrogens may be seen as one of the many variables determining animal fitness for survival. This argument is supported by noting that animal species differ in their sensitivity to phytoestrogens (7). Some species are relatively resistant to the estrogenic effects of these compounds, while others may suffer sterility as a result of prolonged ingestion of phytoestrogens. We have hypothesized that phytoestrogen-induced physiologic and behavioral effects in mammals are significant factors in the reproductive and therefore evolutionary success of the consuming species. We have initiated our analysis of this broad hypothesis by reviewing the available data relevant to the reproductive and general metabolic effects of phytoestrogens in mammals.

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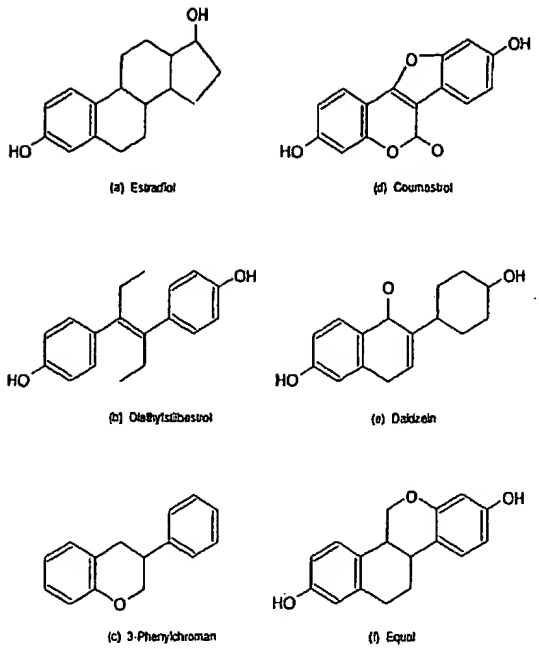

Fig. 1. Structure of common estrogens and phytoestrogens. The major physiological estrogen, estradiol (a), and the potent synthetic estrogen, diethylstilbestrol (b) are shown for reference. 3-Phenylchroman (c) is the phytoestrogen precursor compound to the coumestans such as coumestrol (d) and isoflavones such as daidzein (e). Equol (f) is an estrogenic metabolite produced within the gut from other phytoestrogens of the isoflavone group. (Modified from: Setchell, K. D. R. Naturally Occurring Non-steroidal Estrogens of Dietary Origin. In: Estrogens in the Environment J. A. McLachlan, ed. New York: Elsevier Press, 1985; 69–85.)

PHYTOESTROGEN EXPOSURE

Sources of phytoestrogens

Phytoestrogens are produced by numerous Leguminosae and grasses, including many plants commonly consumed by man and livestock (Table 1). The estrogenic components are found in differing amounts in all parts of the plant, including the seeds, the flowers, the leaves, the roots, and the fruits. Concentrations in each tissue depend on plant type (4,8).

Of particular interest regarding possible human exposure is the presence of phytoestrogens in marijuana and coffee. It had long been suspected that the estrogenic effects of marijuana were due to Δ^9-tetrahydrocannabinol (THC), the major psychoactive compound. Smoking of marijuana significantly suppresses luteinizing hormone (LH) levels

Table 1. Some common plants that contain estrogenic substances

Alfalfa	Coffee	Oats	Rice
Anise	Date Palm	Orchard grass	Rye
Apple	Fennel	Palmetto grass	Sage
Barley	French Beans	Parsley	Sesame
Blue grass	Garlic	Peas	Soybean
Carrot	Green Beans	Pomegranate	Soya sprouts
Cherry	Hops	Potato	Wheat
Clovers	Liquorice	Rape	Yeast
	Marijuana	Red Beans	

during the human menstrual cycle and shortens both the menstrual cycle and the luteal phase (9). Since these results agree with observations in ovariectomized rhesus monkeys injected intramuscularly (i.m.) with THC, it was assumed that the menstrual cycle effects of smoke inhalation would be exclusively due to the THC content of the smoke (10). However, crude marijuana extract and condensed marijuana smoke compete with estradiol for estrogen receptors in the uterus of rats, while in vitro studies detected no binding of cannabinoids to estrogen receptors (11). These findings show that marijuana contains estrogenic substances that may be affecting reproductive processes via cannabinoid-independent mechanisms. Furthermore, apigenin, a derivative of flavonoid phytoestrogens found in crude marijuana, is a moderately potent inhibitor of estradiol binding to uterine estrogen receptors (11). Differentiation between the suppressive effect of THC on LH and the estrogenic effects of marijuana *per se* remains unclear.

Another plant product which is commonly ingested for pleasure rather than nutrition is coffee. Like marijuana, coffee contains weakly estrogenic constituents, evidenced by the estrogenic effects of increased uterine-to-body weight ratio and total uterine protein content following administration of coffee extracts by gavage (12). Ultraviolet absorbance spectroscopy suggests that whatever this active compound may be, it does not belong to one of the three major classes of dietary estrogens (e.g., flavonoids, coumestans, or resorcylic acid lactones). Thus, coffee may contain an estrogen precursor that requires metabolic activation or a structurally unrelated estrogenic compound.

Metabolism, distribution, and clearance

The relative potency of a phytoestrogen depends upon the target tissue, functional state of the target tissue, the animal species involved, and the route and pattern of delivery. In addition, the fami-

lies of estrogenic compounds that occur in plants can be modified by metabolism within the herbivore or even by gut flora prior to uptake. Dietary isoflavone phytoestrogens undergo bacterial modification in the gastrointestinal tracts of animals to yield equol, a weak, nonsteroidal phytoestrogen (8,13,14). Following ingestion of estrogenic plants, a temporary 50- to 1000-fold increase in urinary equol takes place, while insignificant traces of the initially consumed phytoestrogens appear in the urine. Noteworthy is that the major urinary product following the consumption of genistein and biochanin A is p-ethyl phenol, and formononetin consumption yields both daidzein and equol as the major urinary products (4). Furthermore, gut microflora (14) convert daidzein to equol which in turn is absorbed and enters the enterohepatic circulation. Notably, it appears that not all people have the ability to convert other isoflavones to equol. This may be due to the absence of bacteria capable of the conversion of precursors to equol (as is the case in the sterile gut of newborns), the composition (subpopulations) of intestinal microflora present, the intestinal transit time, pH, or redox potential. These factors may be influenced by diet, host immunity, medication use, etc.

Receptor activity and interaction with endogenous estrogens

Phytoestrogens exhibit binding to endogenous estrogen receptors. Binding of phytoestrogens to estrogen receptors is supported by the finding that the larger the dose of phytoestrogen given an organism, the greater the displacement of bound tritiated (^{3}H) E_2 (15). It has also been reported that at very high dosages, all phytoestrogens exhibit more than 80% competitive binding to renal tumor cytosolic estrogen receptors (16). The structural requisites for estrogen receptor binding are met by phytoestrogens. For example, equol possesses a potency on the order of 10^{-3} the estrogenic activity of E_2 and contains a phenyl substituent also present in E_2 and in DES (Figure 1). The substituent considered to be a requirement for estrogenic activity is a hydroxyl group in the same position as the hydroxyl group in the benzene ring of E_2 (14). Another structural similarity which facilitates estrogen receptor binding activity of equol and other phytoestrogens is that the distance between C-3 and C-17 in E_2 is about equal to that between the two hydroxyls in equol.

Considering the large quantities of phytoestrogens ingested by many mammals including man, functionally significant estrogen receptor occupancy by phytoestrogens occurs. Since no phytoestrogen has receptor affinity equal to that of E_2 and the degree of DNA stimulation due to phytoestrogens appears to be substantially less than that evoked by E_2 (8), phytoestrogen actions could be either estrogenic or anti-estrogenic. In a relatively hypoestrogenic individual, receptor occupancy by weak (exogenous) estrogens would likely produce estrogenic effects, while in a normally estrogenized individual, large amounts of weak estrogens might diminish the effective estrogenic activity by competition with E_2.

REPRODUCTIVE EFFECTS IN MAMMALS

Phytoestrogens have been shown to influence virtually every aspect of the mammalian reproductive process via effects on the morphology and physiology of reproductive organs and alteration of sexual behavior. The changes may be reversible or irreversible, depending on the duration and dose of exposure to the phytoestrogens.

Cervix

A pubertal pattern of cell differentiation has been noted in ewes rendered sterile by chronic ingestion of phytoestrogens (17). Among these changes, the cervix assumes a uterine pattern. Folds present in the cervix fuse, resulting in loss of cervical crypts, and the cells of the lamina propria become like those of the uterine stroma. Furthermore, glands having histochemical reactions reminiscent of uterine glands become plentiful in the cervix. Such an increase in abnormal glands may be responsible for the different composition which the cervical mucus takes in sheep with "clover disease." At low phytoestrogen dosage, the cervical mucus has a lower viscosity, not due to a higher water content, but rather due to a decreased concentration of glycoprotein — the component of mucus that affords its consistency. The level of glycoprotein seems to respond to the duration of exposure to the phytoestrogen rather to the dosage of the agent. This change in the cervical mucus compounds the anatomical compromise of the cervix such that the cervical reservoir for sperm in the ewes is greatly reduced. Since sperm recovered from the cervices of clover-affected ewes exhibit decreased motility (17), it appears that the phytoestrogen effect makes the mucus relatively "hostile" in the classic sense of cervical factor infertility. Such spermatoxicity is not understood in general nor in this specific case.

At higher phytoestrogen dosage, both higher volume and water content of cervical mucus are

observed in ewes (17,18), thus indicating that both cervical glycoprotein production and water excretion in the mucus are affected.

The cervical effects of phytoestrogens likely depend upon estrogen receptor mediation. In ewes, phytoestrogen treatment increases the rate of protein and glycoprotein synthesis and the number of estrogen binding sites in the cervix, but binding affinity remains unchanged (19). This finding implies that exogenous estrogen not only occupies the available binding sites, but stimulates the local production of more sites. Such receptor "up-regulation" may make the tissue more sensitive to estrogen action, and, if estrogen exposure continues, the cervical alterations would become more exaggerated.

Uterus

Pronounced uterine effects of phytoestrogens are also observed. The most notable uterine change that occurs is a marked increase in its weight relative to body weight, which constitutes the classic bioassay for estrogen action. A dose-dependent uterine weight increase is precipitated by acute administration of an extract of the Indian herb *Achyranthes aspera* in rats and hamsters at contraceptive dosage (75 mg/kg) and with as little as 1/20 this dosage (20). Similar results have been observed in mice, rats, and hamsters with only 1/40 contraceptive dose of ferujol extract (21). Stob (4) suggests that this hypertrophy of the uterus is the result of "typical estrogenic mechanisms," implying estrogen-receptor mediation. However, a more complex response to daily s.c. injection of female lambs with the phytoestrogen β-sitosterol has been reported, in which uterine weight increases for the first two weeks of treatment but markedly decreases over the next six-week period (22). Plausible explanations for such biphasic results include receptor "down regulation" and induction of metabolic enzymes with enhanced clearance of β-sitosterol. Similar results were obtained using ovariectomized ewes as the model (23).

Another manifestation of the uterotropic effect of phytoestrogens is seen in ewes suffering from infertility due to prolonged exposure to these agents. A marked increase in activity of some uterine enzymes and uterine DNA, protein, and glycoprotein synthesis occurs in such sheep (19). This observation indicates that at least a portion of the uterine weight gain is true hypertrophy rather than simply edema. At the same time, lower levels of lipids within the uteri of sheep fed phytoestrogen suggest inhibition of synthesis or increased utilization of lipids within this organ (22). Thus phytoestrogens may be affecting different enzymes in different fashions, stimulating the activity of some while blocking the action of others. It is noteworthy that the uterine RNA-to-DNA ratio decrease that occurs following ovariectomy is smaller in clover-affected than in normal ewes. This response is accompanied by less regression of the uterus in clover affected ewes than in controls. These findings indicate that phytoestrogenic action may be mediated via differentiations similar to those induced by hormonal steroids during fetal development (24).

Gross structural lesions of the uterus may also result from phytoestrogen exposure and could account for some instances of permanent sterility. Lesser lesions entail the proliferation of cystic endometrium, myometrial fibrosis, and endometrial fibrosis (13). These lesions could certainly compromise normal implantation of the conceptus. The most severe structural failure, complete uterine prolapse, is known to occur in some species following ingestion of some dietary estrogens (mycoestrogens) and obviously disrupts the reproductive process.

It is not clear whether phytoestrogens play any role in pregnancy wastage, but some plant preparations have been used as abortifacients. *Achyranthes aspera,* a common Indian herb claimed to possess abortifacient activity, did induce abortion in mice and rabbits, but failed to show similar effects in rats (20). It is uncertain whether a phytoestrogen is the active agent of *Achyranthes* that brings about abortion, but support for that possibility derives from the finding that miroestrol, a phytoestrogen from a legume tree root, is used by Burmese and Thai women in plant extract form to induce abortion (25). The mechanism for such an abortifacient action of these compounds is unstudied and any effects of phytoestrogens on uterine contractility *per se* have not been determined in either the gravid or nongravid state.

Phytoestrogen effects on uterine function may relate to alterations in activity of several enzymes. Under normal circumstances, oxidative enzymes in the uterus show slight reactions in the endometrium and uterine glands, but after administration of β-sitosterol, these weak reactions are curtailed (22). Such an inhibition of oxidative enzymatic activity in the uterine endometrium and glands may reduce local energy production due to an inability to replenish NAD+ and NADP+. This circumstance would diminish the ability of the uterus to contract and might decrease secretory capabilities of the uterine glands.

Alkaline phosphatase in the uterine tissue of ewes also responds to β-sitosterol in a biphasic pat-

tern. Alkaline phosphatase activity increases over the first two weeks of daily β-sitosterol injections and decreases over the second two weeks of injections (22). This disturbance in alkaline phosphatase activity may alter cell permeability and transport of nutrients by uterine cells.

Acid phosphatase activity in the uterus decreases with increasing dose and time of daily β-sitosterol treatments over an eight-week span (22). Such an inhibition would decrease free phosphorous, and may relate to the more general observation of decreased plasma phosphorus levels in exposed animals.

Uterine cholinesterase activity also decreases following β-sitosterol treatment, as evidenced by its diminished activity towards acetylthiocholine (22). This inhibition of activity is accompanied by a downward shift in sodium ion transport and decreased sodium in the uterine luminal fluid. It is not clear whether effects on sodium transport and cholinesterase activity are coincidental or truly associated processes in this instance.

Ovaries

While many anatomical effects of phytoestrogens have been described, physiologic changes in the reproductive tract are more subtle, but perhaps more consequential. Ovarian cyclicity may be disrupted by phytoestrogen exposure in mammals and birds (2,14,25,26), but interruption of ovulation due to short-term phytoestrogen ingestion is reversible (26). It is plausible that human vegetarians may have ovulatory dysfunction but suffer no other obvious physiologic abnormalities due to their diets (14). Abnormalities of ovulation may be due to direct ovarian actions since administration of β-sitosterol to ewes inhibited follicular development and altered the size distribution of follicles (22). Follicles were observed to show degeneration with intrafollicular hemorrhage and the development of shrivelled oocytes with lipid inclusions. The suggestion of a direct ovarian action of phytoestrogens in perturbing follicular maturation may be supported to some extent by a study which showed that in rats intraperitoneal administration of an extract from a plant species known to contain high concentrations of phytoestrogens inhibited follicular maturation (26). Obviously, these studies cannot distinguish between direct ovarian and indirect effects on follicular growth.

More direct evidence that the follicle may be a site of phytoestrogen activity derives from in vitro cultures of bovine granulosa cells. In this system, lower dosages of genistein and biochanin A increased progesterone synthesis while higher dosages inhibited progesterone synthesis (27). Since progesterone is essential in the establishment and maintenance of pregnancy, such an inhibition of progesterone production would be a plausible explanation for both failure of conception and early pregnancy wastage.

The possibility that phytoestrogens might be toxic to oocytes or early embryos was suggested in a single study (7). Mice fed coumestrol and then mated produced degenerate embryos exhibiting unevenly distributed cytoplasm and lack of symmetry in size among blastomeres, suggesting alterations in cleavage rates. Extensive vacuolization found in the ova also suggests that failure of fertilization of these ova may account for part of the observed decrease in litter size in mice fed coumestrol.

The activities of two ovarian enzymes appear to be influenced by phytoestrogens. First, low doses of phytoestrogen inhibit 17,20-lyase in bovine granulosa cells (27). This effect could profoundly alter the pattern and capacity of the steroidogenic pathways within the follicle or corpus luteum. The precise mechanism by which this effect occurs is unproven. Second, alkaline phosphatase in the ovaries is affected by phytoestrogen exposure (22). While the overall alkaline phosphatase activity is about equal in the ovaries of β-sitosterol-treated and control ewes, the control ewes show an intense reaction in the zona pellucida with a weak reaction in the interstitial tissue. Treated ewes exhibit an opposite response. Thus, a reversal of activities is seen where phytoestrogen is acting both to stimulate and to inhibit the same enzyme in two different sites within the ovary. While a mechanism for this action is not known, such changes in the activities of ovarian enzymes might compromise ovulation and increase the incidence of follicular degeneration in animals treated with phytoestrogens.

CNS/pituitary

Some phytoestrogen effects on ovarian function appear to result from indirect action on the secretion of gonadotropic hormones (7). In this context, there are four possible mechanisms of phytoestrogen action: 1) they are E_2 agonists, 2) they are E_2 antagonists, 3) they act as both E_2 agonists and antagonists, and 4) they act in a nonestrogenic capacity. Available information best supports the third of these possibilities (mixed agonist–antagonist effects). The site of phytoestrogen action could be the CNS (especially hypothalamus), the pituitary, or the gonad (see previous section).

The effect of intraperitoneal injection of phytoestrogen-rich *Dieffenbachia amoena* extract in rats on LH, follicle-stimulating hormone (FSH), prolactin (PRL), progesterone, and E_2 have been studied (26). In treated rats, levels of LH, FSH, and progesterone increased for doses of 2.5, 5.0, and 10.0 mg/kg of extract, while the levels of PRL and E_2 decreased at the same dosages. Progesterone levels showed a biphasic response, increasing at low doses of the extract (26), but decreasing at higher doses (27). Since no obvious single mechanism would explain all of these pituitary and ovarian hormonal changes, the extract may contain more than one endocrinologically active substance, or more than one site or mechanism of action might be involved.

There are data to suggest that phytoestrogens act both at CNS and pituitary levels to alter gonadotropin secretion. In both ovariectomized ewes (23) and intact clover-affected ewes (17), the best explanation for the impairment of gonadotropin secretion was a hypothalamic/CNS action. In particular, in clover-affected ewes, an LH surge could not be elicited by exogenous E_2 administration (consistent with loss of positive feedback), but the LH secretory response to exogenous gonadotropin-releasing hormone was normal (17), suggesting no pituitary effect. Our own data (28) show that acute phytoestrogen administration can alter GnRH-induced LH secretion in ovariectomized rats and thus suggest that the pituitary may be a site of phytoestrogen action in other situations.

Interactions between reproductive effects of phytoestrogen exposure and photoperiod in seasonal breeders have been investigated. In normal intact ewes, the frequency of LH pulses and plasma LH concentration are higher during breeding season than during anestrus season. In clover-diseased ewes, the frequency of LH pulses and LH concentration during breeding season are nearly the same as in normal ewes. In contrast during anestrus season, these LH pulse parameters remain at the high level of breeding season in clover-affected ewes, rather than decreasing as in normal ewes (18). These results suggest that a dissociation of normal photoperiod controls from the LH pulse generator may result from prolonged phytoestrogen exposure.

In ovariectomized ewes given estradiol implants, LH pulse frequency and amplitude vary seasonally, rather like the pattern seen in intact ewes. This seasonal variation in LH pulse frequency in ovariectomized ewes could depend upon extraovarian steroids from the adrenal glands, other intrinsic photoperiod-dependent CNS functional changes, or dietary estrogens. Results from one study suggest that dietary coumestrol decreases the amplitude of LH pulses but fails to affect the frequency of LH pulses or FSH concentrations during the breeding season (23). During anestrus, coumestrol does not alter any of these variables. Thus, coumestrol could only be partially responsible for the seasonal decrease in LH pulse frequency in ewes.

Sexual behavior

Changes in sexual behavior due to phytoestrogen exposure parallel the known physiologic effects. Clover-diseased ewes are slower than normal ewes to exhibit estrus behavior in response to either a single or several daily doses of E_2 (17,29,30). Accompanying the delayed estrus is a retarding of the first mount of the ewes by the ram, although the number of days on which the ewes allowed the ram to mount them does not significantly differ from controls. A delay of estrus in mice fed coumestrol also occurs (7), implying an antiestrogenic effect.

Apparent defeminization of the sexual behavior response following consumption of phytoestrogens is displayed by clover-affected ewes. These ewes show aggressive behavior, such as challenging and head bunting of rams and other ewes, sooner than control ewes following administration of testosterone (17). At the same time the ewes are slower in showing female behavior, such as standing to be mounted by a ram. Furthermore, clover-affected ewes exhibited less soliciting behavior than normals. However, the number of ewes that stood to be mounted decreased equally over the five-week period during which daily testosterone injections were given (30). Relative to controls, clover-diseased ewes exhibit a significantly greater degree of courting behavior 28 but not 21 days following treatment with testosterone. Other courting behaviors that are less hormonally dependent, such as anal and genital sniffing by the ewes, are not altered (17,30). While mechanisms for these behavioral effects are not known, we do know that females and males have similar numbers of estrogen binding sites in the hypothalamus, but estrogen-receptor complexes appear to have shorter nuclear acceptor occupancy in males than in females (31). Behavioral changes in clover-affected ewes could result from a change as simple as a decrease in nuclear acceptor occupancy by estrogen-receptor complexes.

E_2 causes a dose-dependent increase in the incidence and duration of hormone-dependent behaviors in ewes (Table 2), whereas E_2 has no effect on hormone-independent behaviors (30). The E_2-

Table 2. Estradiol-dependent and -independent behaviors in ewes

Hormone-dependent behaviors	Hormone-independent behaviors
Active soliciting	Squatting
Standing for mounting	Looking over shoulder
Allowing ram to mount	Tail fanning
	Kicking

induced behaviors occur less in phytoestrogen-affected ewes than in normals, while E_2 independent behaviors occur with equal frequency in controls and clover-diseased ewes. Since general behavior appears normal but female sex-specific behavior is compromised in phytoestrogen-treated ewes, reproductive success could be compromised on a behavioral basis. The relationship of phytoestrogen-induced anatomic changes in the external genitalia and sexual behavior is not defined, but coital biomechanics could be altered as a result of such end-organ effects. While vulvar and vaginal hypertrophy has been noted in various animals, masculinization has been observed in ewes (17) with clitoromegaly and fusion of the ventral commissure. Upon removal from estrogenic pasture, these changes do not reverse and could, therefore, permanently alter sexual function.

Phytoestrogenic effects in males appear to be consistent with expectations for exogenous administration of bioactive estrogen. Coumestrol increases teat length in wethers (23) and stimulates mammary hypertrophy in intact males. Rams grazed on estrogenic clover have reduced sperm counts (14), but it is not clear whether fertility is affected.

GENERAL METABOLIC EFFECTS IN MAMMALS

Protein synthesis

Some data suggest that phytoestrogens affect levels of plasma proteins. The effects of β-sitosterol on plasma concentrations of albumin, alpha-globulin, beta-globulin, gamma-globulin, and fibrinogen have been studied (32). Normal functions of these proteins are indicated in Table 3 (33). Even though total plasma protein concentration in mice is unaffected by s.c. administration of β-sitosterol, daily 25 to 100 μg injections of the agent increase four of the plasma proteins, but significantly decrease the gamma-globulin complex. The mechanisms of action of phytoestrogens in this system

Table 3. Plasma protein fractions affected by β-sitosterol*

Protein	Function	Effect of β-Sitosterol
Serum albumin	Regulation of blood volume; transport of fatty acids	Increase
Alpha-globulins	Transport of lipids, thyroxine, adrenal cortical hormones, and copper	Increase
Beta-globulins	Transport of lipids, iron, and hemes	Increase
Gamma-globulins	Act as most of the circulating antibodies	Decrease
Fibrinogen	Precursor to fibrin of blood clots	Increase

*(See reference 32).

are not established. It is likely that the phytoestrogens stimulate hepatic protein synthesis but inhibit production of gamma-globulins by lymphoid tissues. It is possible that the increased alpha-globulin concentration is a compensatory occurrence to erythrocyte count reduction that occurs following administration of β-sitosterol, thereby maintaining normal blood viscosity in the absence of normal erythrocyte concentration. The increase in the beta-globulin–fibrinogen complex appears to be correlated with its affinity for binding phosphorus. This affinity increases in response to β-sitosterol (32).

Enzyme activity of the liver

Phytoestrogens influence enzymes in nonreproductive as well as reproductive tissues. A relation between diet and synthesis of three enzymes in the liver of cheetahs has been shown. The affected enzymes, alanine aminotransferase, aspartate aminotransferase, and gamma-glutamyltransferase, decrease in amount when cheetahs are taken off a diet high in soya bean content (thus high in phytoestrogen content) and given a chicken diet (13).

Inorganic plasma constituents

Phytoestrogens induce mineral changes in the blood. Subcutaneous injections of 25, 50, 75, or 100 μg of β-sitosterol increase calcium levels in mice, while doses of 5 or 10 μg of the phytoestrogen have no effect on calcium levels (34). Since E_2 inhibits bone mobilization, β-sitosterol may act by causing a decrease in E_2 levels via inhibition of gonadotropin secretion from the pituitary. Decreased ovarian E_2

production might then result in increased bone mobilization and increased serum calcium. Surprisingly, blood plasma phosphorus levels decrease following administration of 5 to 75 μg doses of β-sitosterol in mice, but show little change in response to a 100 μg dose (34). Decreases in phosphorus could be due to an enhanced rate of storage in an extravascular compartment, increased utilization of phosphorus by tissues, or increased renal clearance.

While β-sitosterol doses of less than 5 μg fail to change plasma magnesium levels, higher doses decrease plasma magnesium and increase both hepatic and intramuscular magnesium (34). Since magnesium is a smooth muscle relaxant, changes in uterine or tubal smooth muscle motility could result indirectly from this phytoestrogen action.

PHYTOESTROGENS IN HUMAN DISEASE

Deleterious roles

Phytoestrogens have been suggested to play both deleterious and beneficial roles with regard to illness. In the diets of cheetahs, phytoestrogens cause vascular hepatic lesions, in which the centrilobular and sublobular hepatic veins are partially or totally occluded (13). The possibility of human hepatic dysfunction must therefore at least be considered.

Vascular disease may be correlated with the consumption of dietary phytoestrogens (35). Coronary heart disease has been suggested to be associated with phytoestrogens consumed indirectly through the milk of cows; that is, the lactating cow consumes the phytoestrogens while grazing and, in turn, phytoestrogens in cow's milk are consumed by humans. One basis for this proposal is that phytoestrogens have more structural similarity to DES, a potent synthetic estrogen found to have atherogenic properties, than to endogenous estrogens such as E_2. The higher rate of coronary heart disease in human males might be explicable in part if human females are found to be better able to metabolize and excrete phytoestrogens.

Dietary estrogens could be a factor in cancer initiation in hormone responsive tissues, but no such instances have been demonstrated. Certainly phytoestrogens bind to both rat and human mammary tumor tissue and show competitive binding for mammary tissue E_2 receptors (15) raising the possibility of stimulation of estrogen-dependent neoplasms.

Beneficial roles

Estrogens have two opposing effects on cancer, depending on dosage. Large doses inhibit breast cancer tumor development and suppress growth of tumors already present, but small doses seem to promote tumor development and stimulate growth (36). This duality extends to phytoestrogens. Phytoestrogens may stimulate or inhibit tumor growth (8,14). One mechanism by which phytoestrogens may manifest their antitumor effects is blockade of estrogen receptors and uncoupling of receptor-mediated response. Thus the ability of endogenous estrogens to support tumor growth would be reduced. Indirect demographic support for a phytoestrogen-mediated reduction in cancers of hormone-responsive tissues might derive from the observation that women in countries consuming vegetarian diets have a lower incidence of breast cancer than in societies where a meat and vegetable diet is consumed (37).

Phytoestrogens may have antiviral and fungicidal properties (37), but a mechanism is not known. Support for the notion that this group of compounds could have such properties may lie in noting that the antifungal drug, ketaconazole, is also a potent inhibitor of some steroidal enzymes.

Plant estrogens have been implicated in the reduction of serum cholesterol levels in humans and animals with hypercholesterolemia. Such action is likely related to the role estrogens play in the metabolism and interaction of lipoproteins with regulation of cholesterol (8).

A final beneficial phytoestrogenic effect is alleviation of vasomotor symptoms in menopausal women. Historically the Chinese have used herbal medicine to treat "hot flushes." These herbal medications work as well as Premarin (an equine conjugated estrogen) in the mitigation of these symptoms in women with natural menopause (38). Similarly, the mycoestrogen, zearalanol, has been reported to reduce the incidence of hot flushes in women with surgical menopause (4). These effects would be consistent with the expected estrogenic properties of these compounds.

CONCLUSION

Phytoestrogens influence mammalian reproductive processes and can thereby compromise the reproductive success of individual mammals and possibly function as a selective environmental factor for populations. While phytoestrogens have a few propitious effects, the majority of the effects are nocuous. These compounds act through their similarity to endogenous estrogens and compete with the endogenous estrogens for binding sites.

Short-term effects of phytoestrogens seem to result from their mixed agonist–antagonist effects on estrogen-mediated processes in mammals. Since long-term exposures can produce persistent, even permanent anatomic, physiologic, or behavioral changes, phytoestrogens must affect the differentiation of some reproductive tissues and irreversibly alter the integration of mammalian reproductive processes in susceptable species.

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Note

Determination of isoflavones in soy bean by high-performance liquid chromatography with amperometric detection

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Soy beans contain several biologically active components including isoflavones such as daidzin, daidzein, genistin, genistein, etc. These isoflavones possess oestrogenic[1], antibacterial[2], antioxidative[3] and spasmolitic[4] activities. Because soy bean protein products are widely used in food products such as infant formulas, health foods and feeds for farm animals, it is necessary to know the concentration of these biologically active components in soy beans. The isolation and quantitation of isoflavones in soy beans have been reported[5–9]. For example, Naim *et al.*[8] developed a gas chromatographic method to isolate and quantitate genistein and daidzein as their trimethyl derivatives, and Murphy[9] reported the separation of daidzin and genistin, and their aglycones, by high-performance liquid chromatography (HPLC) with a gradient of methanol–water. The use of an amperometric detector for HPLC was reported to be useful for the analysis of phenolic compounds such as butylhydroxyanisole, dibutylhydroxytoluene and *tert.*-butylhydroquinone, which are oxidizable[10].

The amperometric determination of daidzin, daidzein, genistin and genistein in defatted soy bean was therefore investigated by HPLC.

EXPERIMENTAL

Genistein was purchased from K & K Labs (Plainview, NY, U.S.A.). Daidzin and daidzein were isolated from *Puerariae radix*[11]. Genistin was isolated as follows. Commercial defatted soy bean flakes were extracted with ethanol for 3 h under reflux, and the extract was partitioned with *n*-butanol and water. The butanol fraction was evaporated under reduced pressure on a rotary evaporator, and subjected to silica gel column chromatography (600 mm × 60 mm I.D., 74–149 μm; Wako, Osaka, Japan) with chloroform–methanol–water (6:1:0.1) in order to obtain a crude genistin fraction. Then, the fraction containing genistin was subjected to preparative HPLC, on a C_{18} column (7 μm, 250 mm × 20 mm I.D.; Yamamura Kagaku, Kyoto, Japan)

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with 30% aqueous acetonitrile as mobile phase. The genistin was recrystallized from 80% ethanol solution: m.p. 256–257°C; $C_{21}H_{20}O_{10}$. IR spectrum in KBr ($\nu_{max.}$, cm^{-1}): 3450, 1661, 1622, 1582, 1180, 1090, 1044, 830. UV spectrum in methanol [$\lambda_{max.}$ (log ε)]: 261 (4.58). Mass spectrum (*m/e*): 284, 270, 166. NMR spectrum in [$^2H_6$]dimethyl sulphoxide (δ, ppm): 12.92 (1H, bs), 9.60 (1H, bs), 8.40 (1H, s), 8.37 (2H, d, J = 7.7 Hz), 7.44 (2H, d, J = 7.7 Hz), 7.34 (1H, d, J = 1.7 Hz), 6.71 (1H, d, J = 1.7 Hz), 5.42 (1H, d, J = 2.2 Hz), 5.30–4.90 (3H, m), 4.30–4.05 (1H, m).

The liquid chromatograph comprised a Shimadzu LC-3A pump (Shimadzu Seisakusho, Kyoto, Japan) equipped with a column oven (Shimadzu CTO-2A) thermostatted at 50°C. The separation of the isoflavones was performed on a reversed-phase column, LiChrosorb RP-8 (5 μm, 250 mm × 4 mm I.D.; Merck) using acetonitrile–0.05 *M* potassium dihydrogenphosphate solution acidified with phosphoric acid to pH 2.0 (15:85) as a mobile phase. The flow-rate was 1.2 ml/min. A Shimadzu SPD-1 spectropohotometer at a wavelength of 260 nm and an IRICA E-502 amperometer (IRICA-Kogyo, Kyoto, Japan), with a glassy carbon working electrode operated at a potential of +0.90 V *vs.* Ag/AgCl, were used in series for the detection.

The sample solution was prepared according to the method of Pettersson and Kiessling[12]. Defatted soy bean flakes (1 g) pulverized with a coffee mill in 25 ml of 80% methanol solution were heated on a water-bath at 80°C for 4 h, and then cooled. A 1-ml volume of the extract was diluted in 3 ml of water and subjected to chromatography on a Waters Sep-Pak C_{18} cartridge (Millipore), which was pre-wetted with methanol and water (each 5 ml). The cartridge was washed with 2 ml of 20% methanol solution and eluted with 2 ml of 80% methanol solution. This eluate was filled up to 5 ml with the HPLC mobile phase for analysis.

RESULTS AND DISCUSSION

Farmakalidis and Murphy[13] reported the use of semi-preparative HPLC with a non-linear gradient of methanol–water for the isolation and purification of the soy bean isoflavones, daidzin and genistin. Fig. 1 shows that an isocratic elution with 30% aqueous methanol is most suitable for separating genistin from other isoflavones. Preliminary experiments showed that the crude soy bean extract could not be directly fractionated by HPLC. Therefore, purification by silica gel column chromatography was necessary before fractionation by preparative HPLC.

Amperometric detection was more effective than ultraviolet (UV) and fluorimetric detection for isoflavones in *Puerariae radix*[10]. In this study, amperometric and UV detectors were used in series in order to cover the wide range of concentration of soy bean isoflavones. The wavelength of the UV detector was set at 260 nm, corresponding to the maximum absorption of genistin and genistein in the mobile phase. To determine the optimum voltage for the amperometric detector, the peak heights of isoflavones were measured at various potentials in the range between +0.60 and +1.10 V *vs.* Ag/AgCl. The peak heights of these compounds increased with increasing potential (Fig. 2). Based on a consideration of the intensity of the background current and the stability of the baseline, the potential of the amperometric detector was set at +0.90 V *vs.* Ag/AgCl.

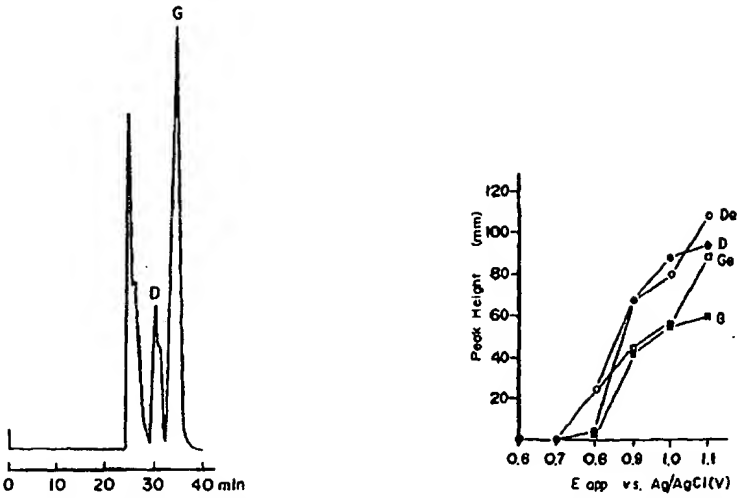

Fig. 1. HPLC of the crude genistin defatted soy bean flakes on a reversed-phase C_{18} semi-preparative column: D = daidzin; G = genistin. Conditions: column, YMC-PACK ODS (7 μm, 250 mm × 20 mm I.D.); mobile phase, water–acetonitrile (70:30); flow-rate, 2.0 ml/min; column temperature, 55°C; detection, UV 260 nm.

Fig. 2. Hydrodynamic voltammograms of daidzin (D), genistin (G), daidzein (De) and genistein (Ge). Conditions: column, LiChrosorb RP-8 (5 μm, 250 mm × 4 mm I.D.); mobile phase, 0.05 *M* potassium dihydrogenphosphate (pH 2.0)–acetonitrile (85:15); flow-rate, 1.2 ml/min; column temperature, 50°C.

To examine the effect of the pH of the phosphate buffer on the peak heights and the capacity factors of these compounds, the pH was changed in the range 2–6. Each peak height was constant. The capacity factors of daidzin and genistin were constant in the pH range studied, while those of daidzein and genistein decreased at pH > 6. On this basis, an acidic phosphate buffer of pH 2.0 was used as the mobile phase. The effect of the concentration of the phosphate buffer on the peak heights and the capacity factors of these isoflavones was also examined in the range 0.01–0.10 *M*. The peak heights and the capacity factors were constant, and the concentration of the phosphate buffer was set at 0.05 *M*.

Under the conditions described above, these compounds were well separated and completely eluted within 32 min. Genistein was detectable at a level of 0.05 ng and other components were detectable at an even lower level. The signal-to-noise ratio (S/N) was 3, and the injection volume was 5 μl. In the range 0.5–75 ng the detector response was linear. As seen in Fig. 3 the detector response towards each compound was higher in amperometric detection than in UV detection. From a chromatogram of a sample solution with a dilution factor of 125 (Fig. 4), the contents of daidzin, genistin, daidzein and genistein in the extract of defatted soy bean flakes were calculated to be 1695, 2935, 131 and 117 μg/g, and their coefficients of variation, from triplicate measurements, were 1.4, 2.5, 3.1 and 3.1%, respectively.

In conclusion, the simultaneous analysis of daidzin, genistin, daidzein and genistein in soy bean by HPLC was achieved. By using an amperometric detector, these

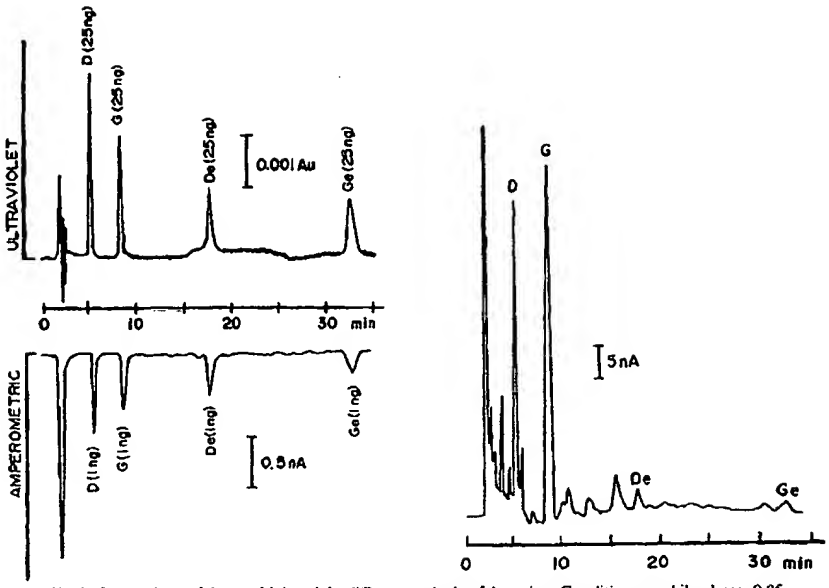

Fig. 3. Comparison of the sensitivity of the different methods of detection. Conditions: mobile phase, 0.05 *M* potassium dihydrogenphosphate (pH 2.0)–acetonitrile (85:15); UV detection, 260 nm (0.02 a.u.f.s.); applied voltage, 0.90 V *vs.* Ag/AgCl (10 nA f.s.).

Fig. 4. Chromatogram of a soy bean extract. Conditions: applied voltage, +0.90 V *vs.* Ag/AgCl; range, 160 nA f.s.

biologically active compounds were sensitively assayed in comparison with UV detection.

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CHROMSYMP. 956

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF PHYTOESTROGENS IN SOY PROTEIN PREPARATIONS WITH ULTRAVIOLET, ELECTROCHEMICAL AND THERMOSPRAY MASS SPECTROMETRIC DETECTION

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SUMMARY

The phytoestrogens daidzein, genistein, coumestrol, formononetin, and Biochanin A are separated on a C_{18} reversed-phase column (Hypersil ODS) with methanol 0.1 *M* ammonium acetate buffer, pH 4.6 (60:40, v/v) as eluent. The retention and resolution are affected by buffer concentrations, pH type, and proportion of organic solvent in the mobile phase. Detection in the (low pg range) is achieved with an electrochemical detector, and the compounds are positively identified by high-performance liquid chromatography–thermospray mass spectrometry. Daidzein and genistein were found in high concentrations in all soy protein preparations analyzed.

INTRODUCTION

The phytoestrogens are a group of naturally occurring plant products[1,2] possessing weak estrogenic activity[3–6]. Their existence in soybeans has been known for some time[7], and recently several phytoestrogens and their metabolites were identified in biological fluids of man[8–11]. In particular, the ingestion of soy protein has been shown to be associated with a vast increase in the urinary excretion of these compounds, and levels in vegetarians generally are higher than those for the general population[8,9,12]. Given the strong association between diet and disease[13], the potential implications, whether beneficial or deleterious, of ingesting biologically active compounds, such as phytoestrogens requires examination. This is particularly the case with the increasing use of soy-based products for human consumption[9], and important to such studies is the requirement of suitable techniques for the detection of these compounds in diets.

Methods for the separation of phytoestrogens in plant extracts by high-performance liquid chromatography (HPLC) have been described[14–22], however, these

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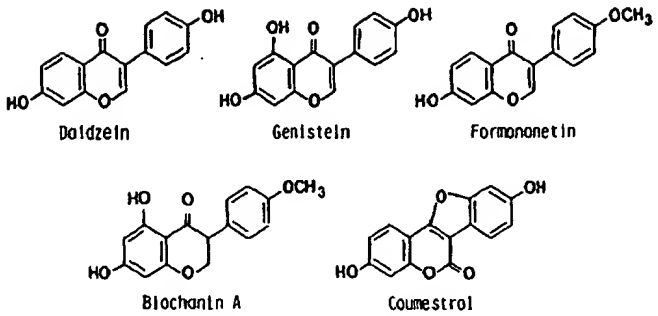

Fig. 1. Chemical structures of the principal plant phytoestrogens.

have generally used gradient elution systems. This paper describes a simple isocratic reversed-phase system, with methanol-0.1 *M* ammonium acetate, pH 4.6 (60:40, v/v) as mobile phase on an Hypersil ODS column, for the rapid and effective separation of the phytoestrogens daidzein, genistein, coumestrol, formononetin, and Biochanin A (Fig. 1). The effects that buffer concentrations, pH, and the type and concentration of organic solvent in the mobile phase have on the retention and resolution have been studied. The sensitivity of ultraviolet (UV) and electrochemical detection (ED) systems has been compared and conditions have been established for HPLC–thermospray mass spectrometry (MS) to allow the identity of individual phytoestrogens in the HPLC effluent to be confirmed. The method has been successfully applied to the analysis of phytoestrogens in a range of soybean products, including soy-based milk formulae and animal diets.

EXPERIMENTAL

Materials and reagents

Biochanin A, genistein, daidzein, and formononetin were from K & K Rare and Fine Chemicals (Plainview, NY, U.S.A.) and coumestrol from Kodak (Rochester, NY, U.S.A.). Textured soy and soy flakes were from Arrowhead Mills, Inc. (Heuford, TX, U.S.A.). Isomil was from Ross Laboratories (Columbus, OH, U.S.A.) and ProSobee was from Mead Johnson (Evansville, IN, U.S.A.). Ammonium acetate, glacial acetic acid, and EDTA were AnalaR-grade from BDH (Poole, U.K.). Acetonitrile and methanol were HPLC grade from Rathburn (Walkerburn, U.K.). The enzyme preparations, β-glucosidase and Helix pomatia (β-glucuronidase and sulfatase) were obtained from Sigma (St. Louis, MO, U.S.A.).

Sample preparation

Samples of Isomil (50 ml), ProSobee (50 ml), or individually homogenized samples of textured soy (5 g) and soy flakes (5 g) were refluxed in 80% aq. ethanol (total volume 250 ml) for 2 h to extract all isoflavones, polar isoflavone conjugates, and related compounds. The organic extracts were cooled, centrifuged, and the supernatant was removed. The ethanol was evaporated in a rotary evaporator, and the lipids were extracted from the remaining aqueous extract by partitioning twice into

four volumes of hexane for textured soy and soy flakes, and partitioning four times for Isomil and ProSobee. The aqueous extract was taken to dryness. Hydrolysis of isoflavone conjugates was carried out using several enzyme preparations. The samples were first subjected to hydrolysis with a β-glucosidase preparation in 0.1 *M* acetate buffer (pH 5.0) overnight at 37°C. The hydrolysate was passed through a cartridge of reversed-phase octadecylsilane-bonded silica (Bond-Elut C_{18}; Analytichem, Harbor City, CA, U.S.A.) to extract all isoflavones, and after washing the cartridge with water, the isoflavones were recovered by elution with 5 ml methanol. After evaporation of the methanol to dryness a second hydrolysis was performed using 0.2 ml of a combined β-glucuronidase and sulfatase preparation (Helix pomatia) in 20 ml 0.5 *M* acetate buffer (pH 4.5) for 24 h at 37°C. The hydrolysate was again passed through a Bond-Elut cartridge to extract the isoflavones, which were recovered by elution with 5 ml methanol and taken to dryness under nitrogen on a 65°C heating block, and the residue was reconstituted prior to assay.

HPLC

A Varian (Walnut Creek, CA, U.S.A.) Model 5000 liquid chromatograph and a Varian UV-100 variable-wavelength detector or a LCA-15 electrochemical detector (EDT Research, London, UK) were used. The electrochemical detector was of the wall-jet type with a glassy-carbon working electrode and a Ag/AgCl reference electrode. Samples were injected via a Rheodyne 7125 injector (Cotati, CA, U.S.A.), fitted with a 100-μl loop.

The column (25 cm × 4.6 mm I.D.) was Hypersil ODS, 5 μm spherical silica, chemically bonded with a monolayer of octadecylsilyl groups from Shandon Southern Products (Runcorn, UK). The mobile phase was methanol–0.1 *M* ammonium acetate buffer, pH 4.6 (60:40, v/v), containing 0.25 mmol/l EDTA. The mobile phase was continuously degassed with a stream of helium during ED. This is unnecessary for UV detection, and EDTA can also be omitted from the mobile phase. The flow-rate was 1 ml/min at ambient temperature and UV detection was at 260 nm. ED of phytoestrogens was achieved at different operating potentials in the range +0.4 to +1.2 V.

Mobile phases of different buffer concentrtions, pH, and with acetonitrile and acetonitrile–methanol mixtures as organic modifiers were used to study their influence on retention and resolution of the individual phytoestrogens.

HPLC–MS

The Varian Model 5000 high-performance liquid chromatograph was coupled to a Finnigan 4635 quadrupole mass spectrometer via a thermospray interface (Finnigan). The mass spectrometer was operated in continuous scanning mode over a mass range of 110–300 daltons. The optimum interface temperatures at the flow-rate used for HPLC separation of the phytoestrogens were determined by multiple injection of standards and varying heater temperatures and repeller voltages. Optimum conditions for the ionization of all the phytoestrogens studied were obtained with a vaporizer temperature of 135°C and a jet-block temperature of 215°C. Solvent flow-rate was 1.0 ml/min, and the HPLC conditions were as described above.

RESULTS AND DISCUSSION

HPLC

The separation of a standard mixture of phytoestrogens on Hypersil ODS with methanol–0.1 *M* ammonium acetate buffer, pH 4.6 (60:40) as eluent is shown in Fig. 2. The elution of these compounds was in the order: daidzein, genistein, coumestrol, formononetin, and Biochanin A. Genistein with three phenolic groups (Fig. 1) is expected to be less hydrophobic than, and therefore eluted before, daidzein, having two phenolic groups. Similarly, Biochanin A with two phenolic groups should, under normal circumstances, be eluted before formononetin with only one phenolic group. The observed reversal in elution order is probably due to the ability of genistein and Biochanin A to form intramolecular hydrogen bonds between one of the phenolic group and the keto group, as shown in Fig. 3. Intramolecular hydrogen bonding will decrease the polarity (increase hydrophobicity) of the molecules, leading to longer retention[21].

Methanol is a better modifier than acetonitrile and is essential for the separation of genistein and coumestrol. The resolution of these two compounds is lost when methanol is replaced with acetonitrile as the modifier (Fig. 2). However, ternary systems which include methanol as one of the organic components will still resolve genistein and coumestrol. For example, a ternary system of acetonitrile–methanol–0.1 *M* ammonium acetate buffer, pH 4.6 (10:50:40) gave a resolution similar to that with the methanol–buffer system.

The pH and buffer concentration of the mobile phase affects the retention but not the resolution. Increasing the pH and/or the buffer concentration decreases the retention of all compounds while maintaining the resolution. A 0.1 *M* buffer at pH

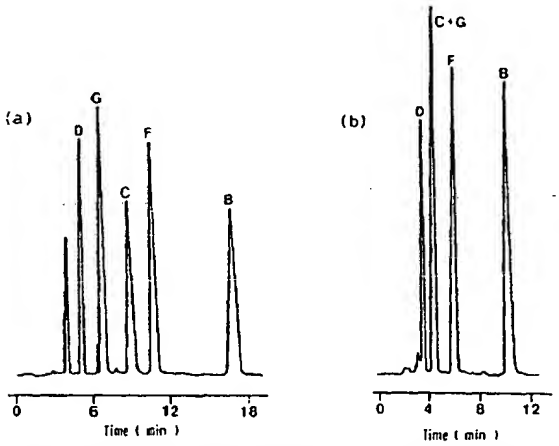

Fig. 2. HPLC separation of phytoestrogen standards, illustrating the effect of varying the mobile phase. (a) Mobile phase: methanol–0.1 *M* ammonium acetate, pH 4.6 (60:40); (b) acetonitrile–0.1 *M* ammonium acetate, pH 4.6 (47:53). Flow-rate 1 ml/min. 260 nm. The following compounds are indicated: D = daidzein; G = genistein; C = coumestrol; F = formononetin; B = Biochanin A.

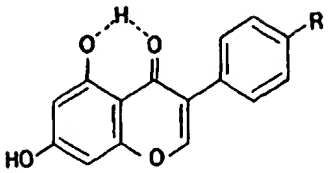

Fig. 3. Intramolecular hydrogen bonding between the phenol and keto groups in the structures of the phytoestrogens genistein (R = OH) and Biochanin A (R = OCH_3).

4.6 was chosen to provide rapid and yet adequate separation of the phytoestrogens, particularly of the early eluted peaks, from possible interferences in sample extracts.

Choice and sensitivity of detector

The phytoestrogens can be detected by UV absorption at 260–280 nm with a detection limit of about 5 ng injected (signal-to-noise ratio of 3 at 0.002 a.u.f.s.). The phytoestrogens are also electroactive, due to the presence of phenolic groups, and can therefore be detected with ED. Coumestrol is the most electroactive compound, followed by genistein and daidzein. The voltammagram for these three compounds is shown in Fig. 4. The optimum potential for the simultaneous sensitive detection of all three compounds is +0.75 V. At a detector sensitivity of 3 nA, the detection limits of coumestrol, genistein and daidzein are 5, 10, and 15 pg injected, respectively. Thus, ED is much more sensitive than the UV detector. However, the satisfactory ED of formononetin and Biochanin A required an operating potential above +1.2 V. At this detector potential, baseline stability becomes a problem. These two compounds are therefore better detected with an UV detector. An UV detector may also be coupled in series with an electrochemical detector for the detection of a wide range of phytoestrogens. However, in preparations containing only daidzein and genistein, ED is the obvious detection system of choice. It allows a much smaller sample size to be used, and therefore a simpler and a cleaner matrix is obtained. For the specific detection of coumestrol a lower operting potential (+0.45 to +0.5 V) may be used, since few compounds are electroactive at these low potentials.

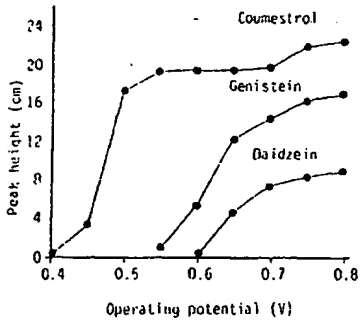

Fig. 4. Voltammagrams for the phytoestrogens coumestrol, genistein and daidzein.

HPLC–thermospray MS

With the development and introduction of the thermospray interface[23–26], many classes of compounds which previously were difficult to analyse by MS can now successfully be analyzed by direct HPLC–MS. Such compounds include those which are highly polar, non-volatile, or thermally labile, such as the phytoestrogens. MS analysis of phytoestrogens in biological fluids has previously necessitated extraction, hydrolysis, purification, and the preparation of volatile derivatives, suitable for introduction into the mass spectrometer via the gas chromatographic outlet[8,10,27,28], techniques which are time consuming.

HPLC–thermospray MS was investigated for its potential to identify individual phytoestrogens in the HPLC effluent under the conditions used here. For all compounds tested the best ionization was achieved at or about a vaporiser temperature of 135°C and a jet-block temperature of 215°C, when the flow-rate was 1 ml/min. Fig. 5 illustrates the total ion current chromatogram, obtained following continuous scanning over the mass range 110–300 *m/z* for a mixture of the five phytoestrogen standards. With the exception of coumestrol, the sensitivity of this technique was comparable to HPLC with UV detection, and no significant loss in chromatographic resolution was observed as a result of interfacing the column with the mass spectrometer. The mass spectra generated in the thermospray ionization process (Fig. 6) were characterized by intense protonated molecular ions, $[MH^+]$, for all of these compounds, and this soft ionization method yielded no significant fragmentation of the molecule.

Since most of the ionization resides in a single ion, selected ion monitoring of the $[MH^+]$ for each phytoestrogen affords a more specific method of detecting these

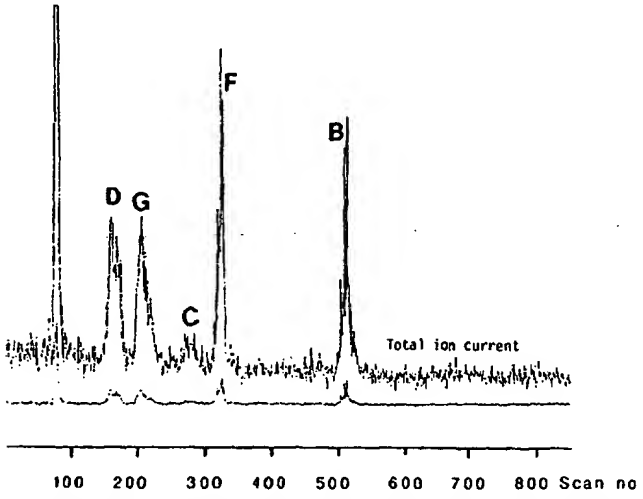

Fig. 5. Total-ion current chromatograms obtained for the HPLC–thermospray MS analysis of a mixture of the phytoestrogen standards listed in Fig. 2.

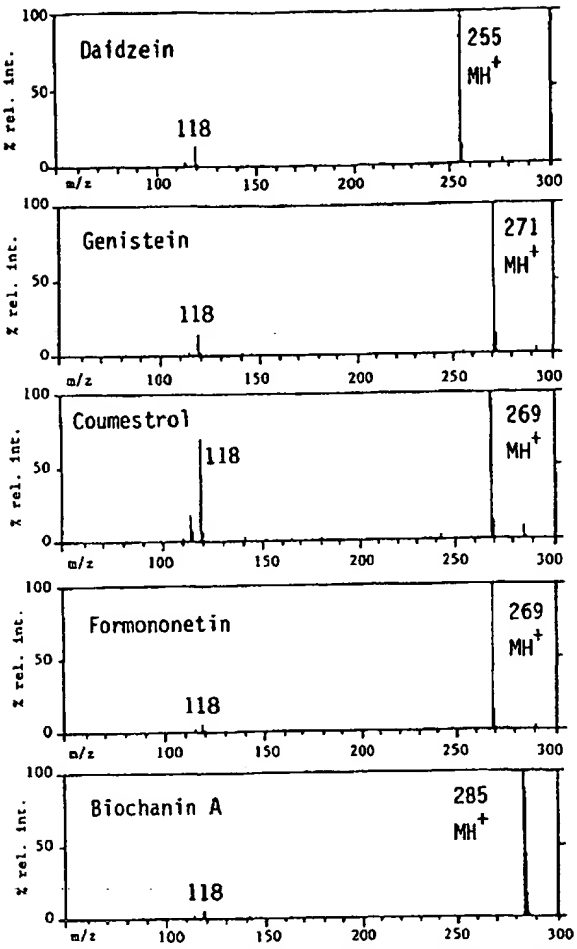

Fig. 6. Mass spectra obtained by thermospray ionization, during HPLC-MS analysis of authentic standards of phytoestrogens.

compounds with a 100-fold improvement in sensitivity over the scanning mode or UV detection alone. Furthermore, we suggest that these compounds would be ideally suited to HPLC–MS–MS detection, where, after focusing the [MH^+] ion, collision-induced dissociation would yield fragmentation specific for each compound, thereby assisting structural elucidation of these and unknown phytoestrogens or metabolites, separated by HPLC. This approach is under evaluation.

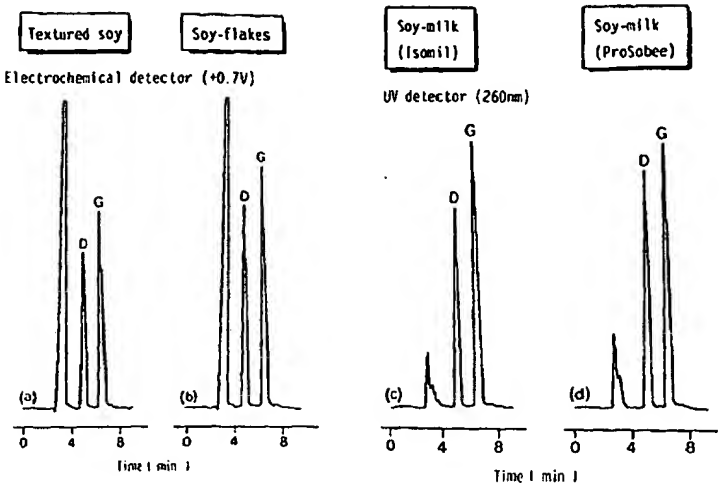

Fig. 7. HPLC profiles of phytoestrogens isolated from samples of soybean products. Both ED and UV detection are illustrated to demonstrate their applicability.

Analysis of soy protein products

Since the phytoestrogens exist in plants mainly as glycoside conjugates[1,2,17,29] or in biological fluids from man and animals as glucuronide or sulphate conjugates[8,10,27], hydrolysis of the conjugate moiety is required prior to HPLC analysis. A general scheme for the analysis of diets or biological fluids was therefore developed to include hydrolysis with glucosidase and/or glucuronidase-sulfatase. Where pure soy protein preparations are to be analyzed, the latter step is unnecessary, but with animal tissues or fluids this step should be considered essential[30]. Ideally, it would be better to develop a system for the direct analysis of the intact conjugates, but at this time the lack of readily available conjugated phytoestrogen standards makes this difficult.

Fig. 7 shows the HPLC analysis for textured soy and soy-flakes by ED and for the soy-milk formulae, Isomil and ProSobee, by UV detection. In all of these soybean products, daidzein and genistein were the only phytoestrogens detected, and

TABLE I

CONCENTRATIONS OF PHYTOESTROGENS IN SOY-BASED PRODUCTS DETERMINED BY HPLC

Soy product	*Daidzein (μg/g)*	*Genistein (μg/g)*
Textured soy	568	568
Soy flake	221	280
Soy-milk formula (ProSobee)	17.1	21.8
Soy-milk formula (Isomil)	19.1	22.6

their concentrations are indicated in Table I. Confirmation of the peaks in each product was made from the mass spectra, obtained by HPLC–thermospray MS, which were identical to those of the authentic compounds.

In earlier reports of the phytoestrogen content of soybean products, daidzein and genistein were the most abundant compounds identified, the latter in slightly higher concentrations[14,15,17–19], but it is evident that there is considerable variability between the different species of soybean and processed products[15].

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MINIREVIEW

Flavones and isoflavones as inducing substances of legume nodulation

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Rhizobia are soil bacteria that can form symbiotic associations with leguminous plants leading to the fixation of atmospheric nitrogen to ammonia which the plant can use. This is an interaction which involves the exchange of many signals between the plant and the bacterium. To start this interaction, rhizobia have adapted to use flavonoid compounds, released by the plant root, as part of a regulatory system to initiate the transcription of their infection (nodulation, *nod*) genes. The development of an assay system for the detection of plant-derived stimulatory biofactors has now led to the isolation and identification of the compounds which are responsible for the activation of the *nod* genes. Stimulatory compounds now have been isolated from plants: from clovers, 7,4'-dihydroxyflavone; from alfalfa, luteolin; from peas, apigenin; and from soybeans, the isoflavones daidzein and genistein. These hydroxylated flavonoid compounds are derived from the phenylpropanoid pathways which are responsible for the synthesis of many important plant phenolic compounds, including the phytoalexin molecules which are thought to be involved in plant defence systems. The current hypothesis on the regulation of the nodulation genes in *Rhizobium* strains is that the gene product of the regulatory *nod* gene, *nodD*, requires the presence of the plant signals to convert it to an active form. This altered NodD protein then induces the expression of the other nodulation genes. This bacterium, induced by plant biofactors, now is able to infect legume root hairs.

Key words: Flavones/isoflavones/nodulation/*Rhizobium*/*nodD*

Introduction

Through a symbiotic association with a soil bacteria [*Rhizobium* or *Bradyrhizobium* (Jordan, 1982)], leguminous plants have the ability to utilize (fix) atmospheric nitrogen gas. Rhizobia are able to induce the formation of morphologically defined structures called nodules on legume roots. The rhizobia within these nodules reduce atmospheric nitrogen to ammonia.

The rhizobia colonize the legume root surface and attach to epidermal and root hair cells (Rovira, 1985). The majority of the bacteria that do so, do not initiate infections of the plant cells. However, some rhizobia specifically interact with newly emerging root hairs and initiate a pronounced curling of these growing hair cells. To initiate this root hair-curling interaction, rhizobia have evolved to use flavonoid compounds released by the plant into the root rhizosphere as regulators of their infection (nodulation, *nod*) genes. Following the initiation of an infection process, rhizobia entrapped within curled root hair cells begin the invasion of these plant cells. Invasion occurs via the induction of an infection thread which penetrates the plant tissue and continues to grow and ramify in the root cortex (Callaham and Torrey, 1981; Turgeon and Bauer, 1982; Ridge and Rolfe, 1985).

The rhizobia trigger cell division within the root and the infection thread eventually invades a focus of dividing plant cells, and rhizobia are released into these cells following 'packaging' within a plant membrane (Robertson and Lyttleton, 1982). The bacteria continue to grow and ultimately differentiate into bacteroids capable of fixing nitrogen (Vincent, 1980). The appropriate physiological environment coupled with some unidentified plant-derived signal is thought to stimulate the depression of the nitrogenase and other bacterial genes involved in nitrogen fixation (Fischer *et al.*, 1986; Regensburger *et al.*, 1986; Ditta *et al.*, 1987).

Studies with legume root exudates

Earlier studies of legume exudates have shown them to both stimulate (Thornton, 1929; Valera and Alexander, 1965; Peters and Alexander, 1966) and inhibit (Nutman, 1953; Turner, 1955) nodulation by rhizobia. Nutman (1953) reported that clover roots excreted a substance inhibitory to nodulation. The substance was not identified but was found to affect an early stage of nodulation on the plant. Turner (1955) later reported that addition of activated charcoal to rooting medium of clover plants inoculated with *Rhizobium* strains led to an increased rate of nodule initiation. Activated charcoal was demonstrated to remove by adsorption an unidentified inhibitory substance secreted by clover roots. In both cases it was suggested that both stimulatory and inhibitory factors were present in the root exudate. Subsequent studies (Valera and Alexander, 1965; Peters and Alexander, 1966) reported a nodulation-enhancing factor in legume exudates that was dialysable, water-soluble and thermostable. This factor could be substituted by coconut water, which contained a factor(s) that influenced the formation of nodules on the roots of *Glycine max* (soybeans) and *Phaseolus vulgaris*. In addition, it was found that an extract of alfalfa seeds could enhance the nodulation of *Medicago sativa*.

Detection of released plant inducing signals

The development of any assay system using a reporter gene for the detection of plant-derived factors has been used to aid in the identification of the compounds which are responsible for the activation of *Rhizobium nod* genes. Fusions of the *Escherichia coli lacZ* gene to *Rhizobium nod* genes have been used to monitor the inducing activity of various plant exudates and extracts (Mulligan and Long, 1985; Innes *et al.*, 1985; Rossen *et al.*, 1985; Zaat *et al.*, 1987). *Rhizobium nod* gene expression is directly coupled to the production of the β-galactosidase enzyme which can be measured easily by colorimetric assays.

Infection of legumes by *Rhizobium* occurs mainly in the emerging root hair zone (Bhuvaneswari *et al.*, 1980; Bauer, 1981). Subsequently, this zone was shown to release substances able to induce *Rhizobium nod* genes by a bioassay system which used seedlings placed on a lawn of *R.trifolii* mutants *nod218* (*nodA:lac* fusion) (Redmond *et al.*, 1986). It was fortuitous that

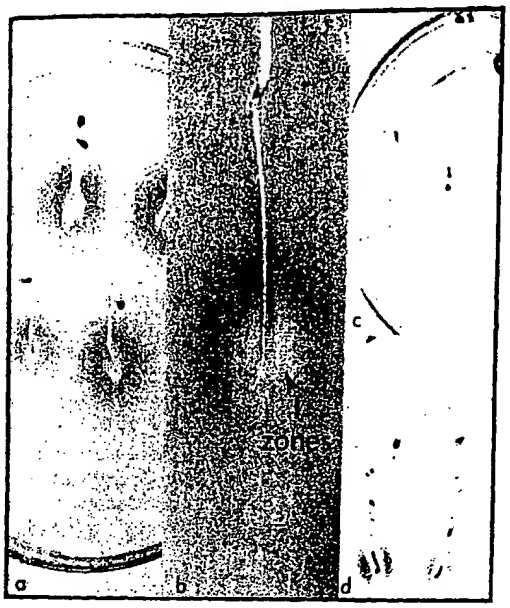

Fig. 1. Demonstration of the release of stimulatory and inhibitory compounds from the root tips of white clovers with a bioassay system showing *nod* gene expression in *R.trifolii* (Redmond *et al.*, 1986; Djordjevic *et al.*, 1987). Axenically grown white clovers or dissected segments of these seedlings were placed on bacterial lawns and incubated at 29°C in the dark for 18 h. (a) White clovers release stimulatory (dark zone) and inhibitory (clear zone) compounds which induce or repress *nodA* 218:*lac* fusion expression in bacteria in the soft agar overlay surrounding the tip. The expression of the *nodA* gene is indicated by the cleavage of X-gal which is incorporated into the soft agar overlay; (b) the expression of the *nodA:lac* fusion occurs only in the vicinity of the roots, the release of the stimulatory and inhibitory compounds appearing from several distinct zones. The centre of the inhibitor zone is near the root tip and for the stimulator the emerging root hair region. As the seedling grows the inhibitor also is released generally over the more mature areas of the root; (c) no expression occurs where a *nodC* anti-sense *lac* fusion was used in the soft agar; (d) dissected segments of white clover seedlings released both stimulatory and inhibitory compounds which suggests that these may be transported systemically throughout the plants.

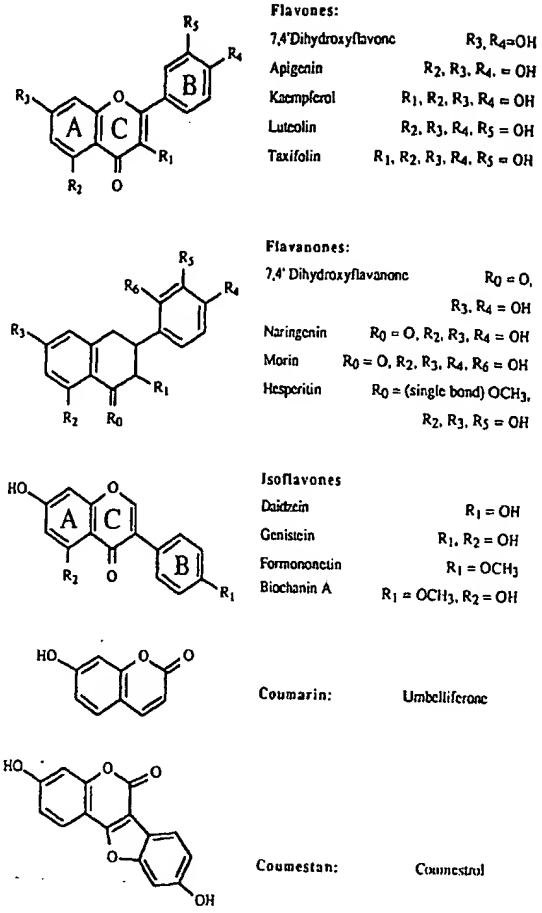

Fig. 2. Structures of the most commonly found plant phenolic compounds which stimulate or inhibit *Rhizobium nod:lac* fusions: the flavones, flavanones, isoflavones, umbelliferone and coumesterol, a coumestan.

the *R.trifolii nod:lac* fusions had the right combination of sensitivity and bacterial genetic background to enable them to be used to visualize the location and timing of the release of plant signals during seedling growth (Figure 1). Expression of *nod* genes in the presence of a plant signal led to production of β-galactosidase and development of a blue colour in the presence of 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal). Intense blue areas extended 10–20 mm from the root tips, cotyledons and cut ends of dissected seedlings (Redmond *et al.*, 1986). Colour was not produced in the absence of either the plants or the bacteria, or when a mutant rhizobia containing a *nod:lac* fusion in the non-sense orientation was used. Within the blue area surrounding the legume root was a distinct, but variable, clear zone which indicated that an inhibition of *nod* gene expression had occurred (Figure 1). Subsequent analysis identified a number of inhibitory substances present in the root exudates of white clovers (Djordjevic *et al.*, 1987). Seedlings of clovers (white, red and subterranean), alfalfa, garden pea var. Rondo, Afghanistan peas, soybean varieties Bragg and Williams, French and broad beans, lupins, siratro and *Desmodium* all released stimulatory factor(s). Some stimulatory compounds are present at very low concentrations in the exudates from tropical legumes which probably explains the induction of the test *nod:lac* fusions.

The non-legumes maize, wheat rice, blue grass var. Kentucky, spinach, cucumber, Brussel sprouts and carrots did not show any detectable stimulatory substances in the bioassays.

Type of plant signals released from legumes

Inducing activities and the probable compounds responsible have been identified or inferred in *Trifolium repens* (white clovers) (Redmond *et al.*, 1986), *Pisum sativa* (peas) (Firmin *et al.*, 1986), *Glycine max* (soybeans) (B.Bassam, M.A.Djordjevic, J.W. Redmond, J.J.Weinman, M.Batley and B.G.Rolfe, in preparation), *Vicia sativa* L. *subsp. nigra* (L.) (Zaat *et al.*, 1987) and *Trifolium pratense* (red clover) (Spaink *et al.*, 1987) (Figure 2 and Table I). In the root exudates and extracts from the temperate legumes (white clovers, alfalfa and peas) flavones and flavanones were identified as the plant-inducing molecules of *Rhizobium nod* genes. For the tropical legumes, such as soybeans, the isoflavones daidzein and genistein were identified as the main inducing substances in the cotyledon and root extracts (Kosslack *et al.*, 1987; B.Bassam, M.A.Djordjevic, J.W.Redmond, M.Batley, J.J.Weinman and R.G.Rolfe, in preparation).

The plant substances which can induce *nod* gene expression within *Rhizobium* cells are derived from the phenylpropanoid pathways (Vickery and Vickery, 1981). These pathways are responsible for the synthesis of many important phenolic com-

Table I. Detected compounds in various legume hosts

Host plant and *nod:lac* assay system	Stimulatory or inhibitory compound	Reference
White clovers[a]	7,4'-dihydroxyflavone (DHF)	Redmond *et al.* (1986)
R.trifolii		
nod218 (*nodA:lac* fusion)	7,4'-dihydroxy-3'-methoxy-flavone (geraldone)	
	4'-hydroxy-7-methoxyflavone isoflavone (formononetin) (I)[c]	Redmond *et al.* (1986)
	7-hydroxycoumarin (umbelliferone) (I)	Djordjevic *et al.* (1987)
Alfalfa[a]		
R.meliloti		
nodC:lacZ fusion pRm57	5,7,3',4'-tetrahyrdoxyflavone (luteolin); low levels of other unidentified flavonoids	Peters *et al.* (1986)
nodD:lacZ fusion pRM61		
Pisum sativa (peas)[b]		
R.leguminosarum		
nodC:lacZ fusion pIJ1477	5,7,3',4'-tetrahydroxyflavanone (eriodictyol),	Firmin *et al.* (1986)
nodD:lacZ fusion pIJ1518	5,4',7-*O*-glucoside trihydroxy-flavone (apigenin-7-*O*-glucoside)	Firmin *et al.* (1986)
	probably apigenin also present in active peakA	Firmin *et al.* (1986)
Vicia sativa L. subsp. *nigra* (L.)[b]		
R.leguminosarum		
Promoter *nodA:lacZ* fusion pMP154	at least 7 different flavanones detected	C.Wijffelman (pers. comm.)
	suggested active molecules: eriodicytol	Zaat *et al.* (1987)
	5,7,4'-trihydroxyflavanone (naringenin),	
	5,7,4'-trihydroxyflavone (apigenin), luteolin	
Glycine max		
(soybeans var. Bragg)[a]		
Strain NGR234		
nod:lac fusion pNGRX6::81	5,7,4'-trihydroxyisoflavone (genistein)	B.Bassam, M.A.Djordjevic, J.W.Redmond M.Batley, J.J.Weinman and B.G.Rolfe (in prep.)
	7,4,-dihydroxyisoflavone (daidzein)	
	plus a small amount of formononetin	
Soybeans var. Williams[b]		
Bradyrhizobium		
strain USDA123	genistein	E.A.Appelbaum (pers. comm.)
nodC:lacZ fusion pEA2-21	daidzein	
Strain USDA110[b]	genistein	G.Stacey (pers. comm.) H.Hennecke (pers. comm.)
nod:lac fusion	daidzein	

[a]Compounds actually identified in exudates and extracts from plants.
[b]Compounds inferred from comparison with commercially available molecules.
[c](I), inhibitory compounds.

pounds in plants, including the phytoalexin molecules which are thought to be associated with plant defence reactions (Figure 3). These inducing molecules are very active even at low concentrations (10^{-7}–10^{-8} M) and can be shown to stimulate *nod* gene expression within minutes of exposure (Djordjevic *et al.*, 1987; Zaat *et al.*, 1987). This great sensitivity of the *Rhizobium nod* gene systems to small amounts of plant signals may reflect the situation in the rhizosphere and indicate the presence of such compounds during root growth.

The flavonoids are chemical structures based on a C15 skeleton with a chromane ring bearing a second aromatic ring (the B-ring) in position 2, 3 or 4 (Figure 2) (Hahlbrock, 1981; Ebel *et al.*, 1986). Flavonoids are classifed into subgroups according to (i) the substitution patterns of the C-ring; (ii) the position of the B-ring; and (iii) the oxidation state of the heterocyclic ring. The major flavonoids are widely distributed in higher plants and their rates of synthesis and degradation vary greatly during different stages of plant development (Hahlbrock, 1981). They function

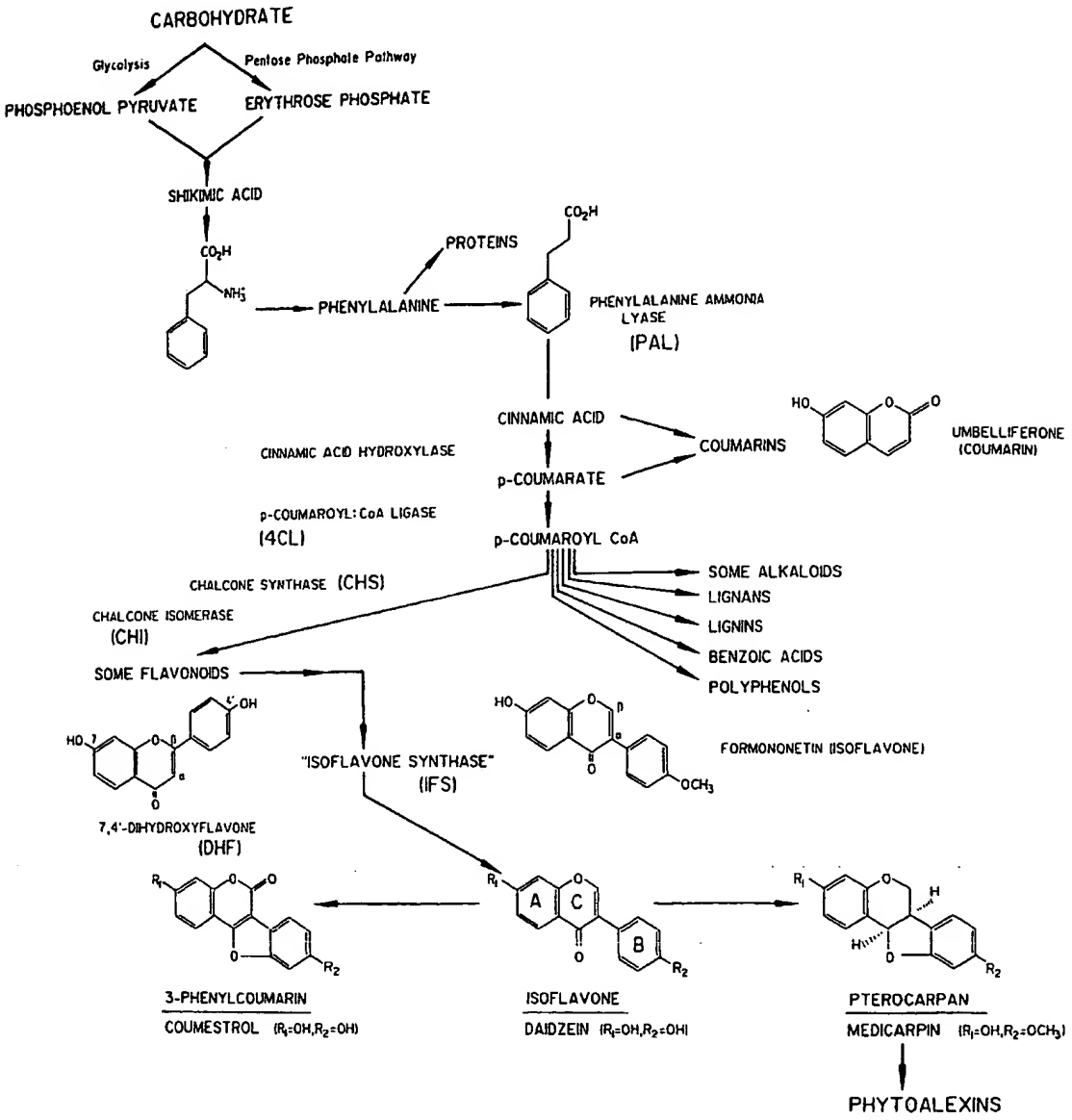

Fig. 3. Proposed phenylpropanoid biosynthetic pathways for shikimic acid and the phenolic plant compounds flavonoids, isoflavonoids and phytoalexins. The flavonoids induce the fast growing rhizobia *R.trifolii*, *R.meliloti* and *R.leguminosarum* while the isoflavonoids induce the fast-growing strain NGR234 and the slow-growing *Bradyrhizobia*. The proposed synthesis of the isoflavones is via a flavonone to an isoflavone which involves a 2,3-aryl migration of the B-ring linked to the C-ring (Hahlbrock, 1981; Ebel *et al.*, 1986). Reproduced with permission from B.G.Rolfe and P.M.Gresshoff (*Ann. Rev. Plant Physiol.*, 39, 1988).

as animal attractants for pollination, and as protective agents against UV light and during infection by phytopathogenic microbes. The isoflavonoids molecules (the B-ring occurs at position 3) have a limited distribution, being restricted mainly to the Leguminosae family (Vickery and Vickery, 1981). However, the isoflavones are important because of their physiological effects on plants and animals. The simple isoflavones, such as formononetin and coumestrol, are phyto-oestrogenic and are responsible for ewe infertility and prolapse of the inverted uterus (Lloyd Davies, 1987). As these occur in leguminous forage plants (red, white and subterranean clovers) they can be of considerable economic importance.

The common breakdown products of flavonoid compounds (hydroxybenzoic acids) had no stimulatory affect (Djordjevic *et al.*, 1987), nor did the *Agrobacterium tumefaciens vir* gene inducer, acetosyringone (Stachel *et al.*, 1985; Yelton *et al.*, 1987), or several glycoslyated flavonoid derivatives, although recently it has been claimed that glucose-*o*-apigenin may have activity (Firmin *et al.*, 1986). As the root exudates from various legumes could stimulate *Rhizobium nod:lac* fusions, it might be inferred that the plant signals do not contribute to host range properties of *Rhizobium* strains. However, as will be described later, it has been shown recently that a combination of particular plant signals and specific NodD gene products do indeed contribute to the phenomenon of host specificity.

Structural requirements of *Rhizobium nod* gene inducers

Induction studies of *nod* gene expression in *Rhizobium* strains established a basic requirement for both a functional *nodD* gene

Table II. Induction of *Rhizodium nod:lac* gene fusions by commercially available compounds

Compound	*Rhizobium* test strain			
	R.trifolii	*R.leguminosarum*	*R.meliloti*	NGR234
7,4'-Dihydroxyflavone (DHF)	+			+
6-OH-flavone	−			−
7-OH-flavone	+	±	−	+
3-OH-flavone	−			−
5,7-DiOH-flavone (chrysin)	+	−	−	+
5-OH-flavone	−	−		
4'-OH-7-methoxyflavone	+			
Geraldone	+			
Apigenin	+	+	±	+
Luteolin	+	+	+	+
7,3',4'-Tri-OH-flavone		+		
7,4'-DiOH-flavanone	+			+
5,7,3',4'-Tetra-OH-flavone (inteolin)		+		
Naringenin	+	+	+	+
Eriodictyol	NT	+	+	NT
Apigenin monoglucoside	+	+		+
3,5,7,4'-Tetra-OH-flavone (kaempferol)	±	−		+
5,7,6',8'-Tetra-OH-flavone (morin)	−		−	
3,5,7,3',4'-Penta-OH-flavone (quercetin)	±	−	−	+
3,7,3',4'-Tetra-OH-dihydroflavone (fisetin)	−			
3,5,7,3',4',5'-Hexa-OH-falvone	−			
7,4'-DiOH-flavone-8-C-glucoside (bayin)	−			
3,5,7,3',4'-Penta-OH-3-*O*-rutinoside (rutin)	±			
5,3'-DiOH-4'-methoxyflavone-7-*O*-rutinoside (diosmin)	−			
3,5,7,3',4'-Penta-OH-dihydroflavone (taxifolin)	±			±
Myricetin	−			−
5,7,3'-Tri-OH-4'-methoxyflavone (hesperitin)	−	+		+
5,7,4'-Tri-OH-flavanone-7-*O*-rhamnoglucoside (naringin)	−	−		
Isoflavones				
Formononetin	−	−		+
Daidzein	−	−		+
Genistein	−	−		+
Biochanin A	−			+
Coumestrol	−			+
Coumarins				
Coumarin	−			−
7-OH-coumarin (umbelliferone)	−			+

A + sign indicates induction of *nodA:lac* gene fusions; a ± indicates weak induction; a − indicates no induction of *nodA:lac* gene fusions; induction is assayed as β-galactosidase activity using X-gal or ONPG methods; NT, not tested. Taxifolin, naringenin and naringin are flavanones (dihydroflavones) in which the double bond between carbons 2 and 3 of the fused (C ring) ring is saturated (see structure, Figure 2).

(Mulligan and Long, 1985; Rossen *et al.*, 1985) and an appropriate plant signal (Innes *et al.*, 1985; Peters *et al.*, 1986; Redmond *et al.*, 1986; Firmin *et al.*, 1986; Spaink *et al.*, 1987; Zaat *et al.*, 1987). Furthermore, these investigations tested the inducing activities of a variety of flavonoid and isoflavonoid compounds and suggested structural features of the A-, B- and C-rings that were required for inducing activity. The compounds which produced a significant enhancement of the expression of the *nod:lac* fusions were all compounds with a hydroxyl group at the 7-position on the A-ring (Table II and Figure 2). This finding was true for the *R.trifolii*, *R.leguminosarum* and *R.meliloti* test systems. However, considerable differences have been found between the reactions of the NodD gene products from the different *Rhizobium* strains (Table II; Spaink *et al.*, 1987). The NodD gene product of *R.leguminosarum* shows a greater preference to the flavones than the NodD1 gene product of *R.meliloti*. The *R.trifolii* NodD gene product responds to a broader spectrum of flavonoid and flavanoid compounds (Table II), while the NodD gene product of strain NGR234 is able to interact with a large number of flavonoid, flavanoid and isoflavonoid molecules to cause *nod* gene induction.

The presence of a 3-hydroxyl group on the test compound (kaempferol, fisetin, quercetin, flavonol, morin and myricetin) reduces the inducible activity for *R.trifolii nod:lac* fusions to low levels and fails to induce *R.meliloti nod* genes. The *R.meliloti* NodD1 gene product also requires that the inducing molecules have hydroxylation at the 3' and/or 4' positions of the B-ring (Peters *et al.*, 1986). Isoflavones (the B-ring located on the C3 position of the C-ring) (biochanin A, genistein, daidzein, formononetin) and coumarins (umbelliferone, a 7-OH coumarin and coumarin) had no stimulatory effect on the *nod* genes of *R.trifolii*, *R.leguminosarum* and *R.meliloti*. In contrast, the expression of the *nod* genes of strain NGR234 can be readily induced by these molecules (Table II).

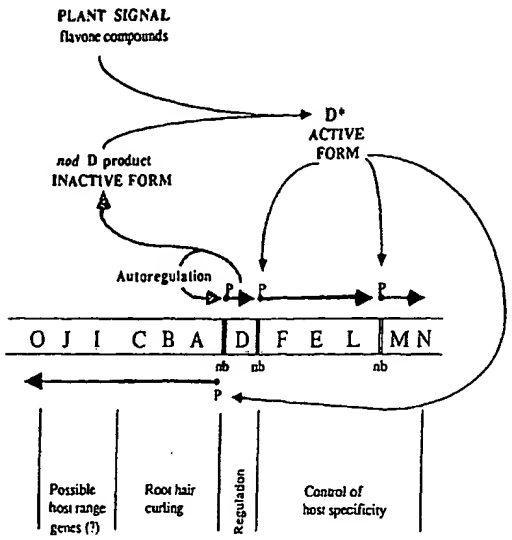

Fig. 4. Current model of the regulation of the cluster of the different operons of nodulation genes of *R.trifolii*. The *nodD* gene is autoregulated via the NodD gene product in *R.trifolii* (MA.Djordjevic, J.J.Weinman and B.G.Rolfe, in preparation) and *R.leguminosarum* (Rossen *et al.*, 1985), but the *R.meliloti nodD* gene is not (C.Wijffelman, personal communication). It is proposed that the inactive form of the NodD gene product (Mulligan and Long, 1985; Rossen *et al.*, 1985) is activated by the interaction with a plant-derived signal (for white clovers, DHF) which now can lead to the initiation of the expression of the *nodABCIJO*, *nodFEL* and *nodMN* operons. The *nodO* gene is a newly identified gene, probably involved in Afghanistan pea nodulation (J.M.Watson, personal communication). The various operons affect different phenotypes, such as root hair curling, host specificity or overall regulation of the nodulation genes.

Inhibitory compounds repress the action of stimulatory compounds

When the extracts from 500 000 white clover seedlings were characterized three active components were found which could induce *Rhizobium nod:lac* fusions (Redmond *et al.*, 1986). Two other substances were present in the extracts at about 3–4 times the concentration of the active flavones and were identified as the isoflavone formononetin and the 7-OH coumarin umbelliferone (Djordjevic *et al.*, 1987). Subsequent experiments showed that both these compounds could inhibit the induction of *R.trifolii nod* gene fusions by stimulatory compounds. Furthermore, these *in vitro* experiments show that the activation of *nod* genes is determined by the ratio of stimulator to inhibitor. The inhibitory mechanism appears to be competitive and the target for the action of the inhibitory and stimulatory compounds is either the NodD product or a common target which is likely to be membrane-bound.

Chrysin (a flavone) and kaempferol (a flavonol) caused significantly lower levels of induction of *R.trifolii nod:lac* fusions than 7,4'-dihydroxyflavone (DHF), but were active in a similar concentration range (Djordjevic *et al.*, 1987). When these two molecules and the isoflavones formononetin, daidzein, biochanin A and umbelliferone (which produced no stimulatory activity themselves) were tested in competition experiments with the stimulatory DHF, they could inhibit *nod* gene induction. It was concluded that these substances probably bind to the same site as DHF and hence can behave as antagonists of *nod* gene induction. All compounds that were able to compete with 5×10^{-7} M DHF and reduce the level of induced gene transcription had molecular structures with a 7-OH substituent (Djordjevic *et al.*, 1987). The presence of a glucose molecule near this C-7-OH position, as found in bayin, a 7,4'-diOH flavone-8-C-glucoside, blocks the stimulatory activity of the DHF molecule. However, bayin is an effective antagonist of *R.trifolii nod* gene expression, so it may still compete with DHF for active sites.

Unidentified inhibitory compounds also have been detected in pea root exudates that antagonize the induction of the *R.leguminosarum nodC:lacZ* fusion (Firmin *et al.*, 1986). These workers tested the antagonistic effect of selected flavoid and acetophenone analogues on *R.leguminosarum nod* gene expression. Chrysin, kaempferol, daidzein and genistein (an isoflavone) all inhibited induction but so did the flavonoid compound 3,3',4',5-tetra-OH-7-methoxyflavone (rhamnetin) and the acetophenone analogues which had a substitution similar to that found in the B-ring. It is interesting that the isoflavones are the inducing molecules for *nod* gene expression in *Bradyrhizobium* strains and the fast-growing *Rhizobium* strain NGR234 but are inhibitory to the more narrow host range rhizobia *R.trifolii* and *R.leguminosarum*.

The release of the inhibitory compounds varies over the developing clover root, as does the production of the stimulator compounds (Figure 1). While the stimulator is chiefly released from plant cells behind the growing root tip in the zone of emerging root hairs, the release of the inhibitor occurs in the region between this zone and the root tip and elsewhere on the root (Figure 1). The plant cells behind the root tip have been shown to be the most susceptible cells to infection (Bchuvaneswari *et al.*, 1980), so it is probably not a coincidence that this constitutes the major site of the release of stimulatory compounds. The chief determinant of the sites of nodule initiation could therefore be the ratio of stimulator:inhibitor in the vicinity of potential infection sites or inside the induced infection thread. Fluctuations in the concentrations of the stimulatory and inhibitory compounds in the plant will affect the expression of the *nod* genes in the infection thread. An excess of inhibitory compounds may cause the infection thread to cease because the bacteria are not able to continually stimulate the plant to (i) continue synthesis of the infection thread cell wall or (ii) maintain continuous cortical cell divisions. Thus regulation of flavone, coumarin and isoflavone synthesis may be a mechanism by which legumes prevent over-nodulation. The inhibitory compounds could be the rapidly induced systematic factor(s) which control nodule number (Nutman, 1952, 1967; Pierce and Bauer, 1983). Furthermore, the 'supernodulating' soybean (Carroll *et al.*, 1985) or pea (Jacobsen and Feenstra, 1984) mutants might be explained by the loss or reduction of inhibitor synthesis at the sites of infection initiation.

Genetic organization of *Rhizobium* genes affecting nodulation

The genetic determinants for *Rhizobium* invasion of a plant host are located both on the bacterial chromosome and indigenous plasmids. A clustering of nodulation genes occurs on one particular plasmid known as the symbiotic (Sym) plasmid (Banfalvi *et al.*, 1981; Djordjevic *et al.*, 1983). Those *nod* genes so far defined (designated *nodABCDEFIJLMN*) in *R.trifolii* and *R.leguminosarum* strains are arranged in four separate operons (*nodABCIJO*, *nodD*, *nodFEL* and *nodMN*), based on DNA sequence analysis, complementation data and expression studies using *nod* genes fused to *E.coli* genes (Rossen *et al.*, 1984;

Schofield *et al.*, 1984; Torok *et al.*, 1984; Egelhoff *et al.*, 1985; Djordjevic *et al.*, 1985, 1986; Schofield and Watson, 1986; Shearman *et al.*, 1986; Fisher *et al.*, 1987). The main regulatory gene, the *nodD* gene, in many rhizobia is linked to the *nodABC* genes but is transcribed divergently. The NodD gene product is thought to be a type of 'environmental sieve', detecting the concentrations of stimulatory and inhibitory compounds released by a particular legume. Some species of *Rhizobium* contain multiple copies of the *nodD* gene, which suggests that specific *nodD*–plant signal interactions may occur. The expression of the *nodD* gene has been shown to be constitutive (Mulligan and Long, 1985; Rossen *et al.*, 1985; Innes *et al.*, 1985).

The current working hypothesis on the regulation of the nodulation genes in *Rhizobium* strains is that the NodD gene product requires the presence of substances secreted by the plant host to convert it to an active form (Mulligan and Long, 1985; Rossen *et al.*, 1985). This altered NodD protein then induces the expression of the genes in *nodABCIJO*, *nodFEL* and *nodMN* operons (Rostas *et al.*, 1986) (Figure 4). While this is an attractive model it still has to be established that (i) flavones or other stimulators can enter the *Rhizobium* cell; (ii) that they associate with the NodD gene product in some way; and (iii) that his 'modified *nodD*–stimulator' complex can bind to *Rhizobium* promoters in the various species.

Additional support for this model of the interaction of the NodD gene product with a plant signal comes from the isolation of a series of *nodD* mutants of *R.trifolii* which have a higher constitutive expression of the *nodABCIJO* operon (J.J.Weinman, M.A.Djordjevic, J.W.Redmond, M.Batley, B.G.Rolfe, in preparation). These mutants are now stimulated by the original inhibitors (coumarin umbelliferone; isoflavones biochanin A, daidzein and formononetin) and partially inhibited by the previous stimulatory compounds (7,4'-dihydroxyflavone, chrysin and luteolin). The isolation of such *nodD* mutants, reversed in their responses to the original stimulatory and inhibitory compounds, is more easily explained if these plant signals directly interact with the NodD gene product. Collectively, the data argue that the conformation of the NodD protein would confer specificity to a particular set of stimulator or inhibitor compounds.

Summary

In the intricate interaction between rhizobia and their host plants, the first most crucial step is that the appropriate NodD protein–flavonoid/isoflavonoid complex is formed so that the initiation of transcription of the other nodulation genes may begin. It still remains, however, to be established that these plant derived molecules can pass into *Rhizobium* cells and that they are not processed in any way before the activation of the NodD gene product can take place. It is now clear that the amino acid sequence of the resident NodD gene product of a particular *Rhizobium* strain determines whether flavones, flavanones, flavonols, isoflavones or umbelliferone act as stimulators or inhibitors. Furthermore, different legumes release different sets of compounds, and some will be perceived as stimulatory and others as antagonistic, and this assessment of these rhizosphere plant signals is one of the important functions of the *Rhizobium* NodD gene product. There is a paradox of why the narrow host-range rhizobia, *R.trifolii*, *R.meliloti* and *R.leguminosarum*, have adapted to respond to the flavones and flavanones rather than the more prevalent isoflavones which are found mainly in the Leguminosae family. This might be explained in terms of the regulation of the biosynthesis of the isoflavones and phytoalexins, which is thought to occur via a flavonone to isoflavone step (Ebel *et al.*, 1986). Both these substances can exhibit anti-microbial activity and excessive amounts of these molecules during *Rhizobium* infection could be detrimental. Regulation of the biosynthesis of these molecules in the temperate legumes (clovers, peas, alfalfa) could be different to that in the tropical legumes. Future studies of the regulation of the phenylpropanoid pathways in these plants should provide some insight to this problem and to the other roles that these biofactors may play in the biology of the microbe–plant interactions.

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